FACULTY OF VETERINARY MEDICINE

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MICROBIOLOGY AND EPIDEMIOLOGY

DETECTION, EPIDEMIOLOGY AND HOST SPECTRUM OF COWPOX AND BORNA DISEASE VIRUS INFECTIONS

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ABSTRACT

Several orthopoxviruses (OPV) and Borna disease virus (BDV) are enveloped, zoonotic viruses with a wide geographical distribution. OPV antibodies cross-react, and former smallpox vaccination has therefore protected human populations from another OPV infection, rodent-borne cowpox virus (CPXV). Cowpox in humans and cats usually manifests as a mild, self-limiting dermatitis and constitutional symptoms, but it can be severe and even life-threatening in the immunocompromised. Classical Borna disease is a progressive meningoencephalomyelitis in horses and sheep known in central Europe for centuries. Nowadays the virus or its close relative infects humans and also several other species in central Europe and elsewhere, but the existence of human Borna disease with its suspected neuropsychiatric symptoms is controversial. The epidemiology of BDV is largely unknown, and the present situation is even more intriguing following the recent detection of several-million-year-old, endogenized BDV genes in primate and various other vertebrate genomes.

The aims of this study were to elucidate the importance of CPXV and BDV in Finland and in possible host species, and particularly to 1) establish relevant methods for the detection of CPXV and other OPVs as well as BDV in Finland, 2) determine whether CPXV and BDV exist in Finland, 3) discover how common OPV immunity is in different age groups in Finland, 4) characterize possible disease cases and clarify their epidemiological context, 5) establish the hosts and possible reservoir species of these viruses and their geographical distribution in wild rodents, and 6) elucidate the infection kinetics of BDV in the bank vole.

An indirect immunofluorescence assay and avidity measurement were established for the detection, timing and verification of OPV or BDV antibodies in thousands of blood samples from humans, horses, ruminants, lynxes, gallinaceous birds, dogs, cats and rodents. The mostly vaccine-derived OPV seroprevalence was found to decrease gradually according to the year of birth of the sampled human subjects from 100% to 10% in those born after 1977. On the other hand, OPV antibodies indicating natural contact with CPXV or other OPVs were commonly found in domestic and wild animals: the horse, cow, lynx, dog, cat and, with a prevalence occasionally even as high as 92%, in wild rodents, including some previously undetected species and new regions. Antibodies to BDV were detected in humans, horses, a dog, cats, and for the first time in wild rodents, such as bank voles (*Myodes glareolus*). Because of the controversy within the human Borna disease field, extra verification methods were established for BDV antibody findings: recombinant nucleocapsid and phosphoproteins were produced in *Escherichia coli* and in a baculovirus system, and peptide arrays were additionally applied. With these verification assays, Finnish human, equine, feline and rodent BDV infections were confirmed. Taken together, wide host spectra were

evident for both OPV and BDV infections based on the antibody findings, and OPV infections were found to be geographically broadly distributed.

PCR amplification methods were utilised for hundreds of blood and tissue samples. The methods included conventional, nested and real-time PCRs with or without the reverse transcription step and detecting four or two genes of OPVs and BDV, respectively. OPV DNA could be amplified from two human patients and three bank voles, whereas no BDV RNA was detected in naturally infected individuals. Based on the phylogenetic analyses, the Finnish OPV sequences were closely related although not identical to a Russian CPXV isolate, and clearly different from other CPXV strains. Moreover, the Finnish sequences only equalled each other, but the short amplicons obtained from German rodents were identical to monkeypox virus, in addition to German CPXV variants. This reflects the close relationship of all OPVs. In summary, RNA of the Finnish BDV variant could not be detected with the available PCR methods, but OPV DNA infrequently could. The OPV species infecting the patients of this study was proven to be CPXV, which is most probably also responsible for the rodent infections.

Multiple cell lines and some newborn rodents were utilised in the isolation of CPXV and BDV from patient and wildlife samples. CPXV could be isolated from a child with severe, generalised cowpox. BDV isolation attempts from rodents were unsuccessful in this study. However, in parallel studies, a transient BDV infection of cells inoculated with equine brain material was detected, and BDV antigens discovered in archival animal brains using established immunohistology. Thus, based on several independent methods, both CPXV and BDV (or a closely related agent) were shown to be present in Finland.

Bank voles could be productively infected with BDV. This experimental infection did not result in notable pathological findings or symptoms, despite the intense spread of the virus in the central and peripheral nervous system. Infected voles commonly excreted the virus in urine and faeces, which emphasises their possible role as a BDV reservoir. Moreover, BDV RNA was regularly reverse transcribed into DNA in bank voles, which was detected by amplifying DNA by PCR without reverse transcription, and verified with nuclease treatments. This finding indicates that BDV genes could be endogenized during an acute infection. Although further transmission studies are needed, this experimental infection demonstrated that the bank vole can function as a potential BDV reservoir.

In summary, multiple methods were established and applied in large panels to detect two zoonoses novel to Finland: cowpox virus and Borna disease virus. Moreover, new information was obtained on their geographical distribution, host spectrum, epidemiology and infection kinetics.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to by their Roman numerals.

I Pelkonen PM, Tarvainen K, Hynninen A, Kallio ER, Henttonen H, Palva A, Vaheri A, and Vapalahti O. 2003. Cowpox with severe generalized eruption, Finland. Emerging Infectious Diseases 9: 1458-1461.

II Kinnunen PM, Henttonen H, Hoffmann B, Kallio ER, Korthase C, Laakkonen J, Niemimaa J, Palva A, Schlegel M, Sheikh Ali H, Suominen P, Ulrich RG, Vaheri A, and Vapalahti O. 2011. Orthopoxvirus infections in Eurasian wild rodents. Vector-Borne and Zoonotic Diseases. doi:10.1089/vbz.2010.0170

III Kinnunen PM, Billich C, Ek-Kommonen C, Henttonen H, Kallio ER, Niemimaa J, Palva A, Staeheli P, Vaheri A and Vapalahti O. 2007. Serological evidence for Borna disease virus infection in humans, wild rodents and other vertebrates in Finland. Journal of Clinical Virology 38: 64-9.

IV Kinnunen PM, Inkeroinen H, Ilander M, Kallio ER, Heikkilä HP, Koskela E, Mappes T, Palva A, Vaheri A, Kipar A, and Vapalahti O. Intracerebral Borna disease virus infection of bank voles leading to peripheral spread and reverse transcription of viral RNA. Submitted.

 In addition, some unpublished data are presented.

ABBREVIATIONS

ABV	avian bornavirus
AIX	avidity index = (antibody titre with urea wash) / (antibody titre without urea wash) x 100%
BD	Borna disease
BDV	Borna disease virus
bp	base pairs
C6	rat astrocyte cell line
cDNA	complementary DNA
CIC	circulating immunocomplex
CMLV	camelpox virus
CNS	central nervous system
CPXV	cowpox virus
CSF	cerebrospinal fluid
C_t	cycle threshold in real-time PCR
dpi	days post infection
EBL	endogenous Borna-like element
EBLN	endogenous Borna-like N element
ECTV	ectromelia virus
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
ffu	focus-forming unit
HA	haemagglutinin
He/80	Borna virus strain “Herzog 1980”

i.c.	intracerebral
IF	immunofluorescent
IFA	indirect immunofluorescence assay
Ig	immunoglobulin
kb	kilobase, 1000 base pairs
kDa	kilodalton
L	RNA polymerase of Borna disease virus
M	matrix protein
MPXV	monkeypox virus
N	nucleocapsid protein
OPV	orthopoxvirus
P	phosphoprotein
PBS	phosphate-buffered saline
PC	Purkinje cell
PCR	polymerase chain reaction
p.i.	post infection
REB	rabbit embryonic brain cells
RNP	ribonucleoprotein
RT	reverse transcriptase / reverse transcription
*Sf*9	*Spodoptera frugiperda* insect´s cells
VACV	vaccinia virus
VARV	variola / smallpox virus

1. INTRODUCTION

Any infection or disease that is naturally transmissible between vertebrate animals and humans is, according to the definition of the World Health Organization, classified as a zoonosis (3). More than 60% of all human diseases and as many as 80% of emerging ones are zoonoses (66). Of the emerging zoonotic pathogens, 58% infect ungulates, 51% carnivores, and 34% rodents emphasising the utmost importance of collaboration between veterinary, medical and wildlife sciences in the detection and battle against these threats. Viruses are the most ubiquitous emerging pathogens, as they cause about half of emerging diseases in humans, domestic livestock and domestic carnivores (66).

This thesis addresses to two zoonotic, probably emerging viruses: cowpox virus (CPXV) and Borna disease virus (BDV). Both are well known veterinary pathogens that, in addition to ungulates and carnivores, also infect humans and have their proven (CPXV) or suspected (BDV) host among wild rodents (63, 299). CPXV infection in domestic animals (and especially cats, despite the name) and humans leads to cowpox, the signs of which vary from painful dermatitis to severe, even lethal systemic infection (28). Animal Borna disease has classically been described as a chronic, progressive meningoencephalomyelitis including both neurological and behavioural symptoms in horses and sheep (92). During recent decades, milder manifestations and a wider host range have also been seen (34, 196). In humans, numerous neuropsychiatric entities have been associated with BDV infection, but the causative role is controversial (262, 266). This view has become even more intriguing since the recent observation of BDV gene endogenization in vertebrate genomes (27, 155).

CPXV belongs to orthopoxviruses (OPV) together with other related, cross-reactive and cross-protective viruses such as vaccinia virus (VACV) and variola virus (VARV), the latter having caused smallpox, the most serious disease of humankind (102). The cross-protection has been utilised in the eradication of smallpox ever since the 18th century, when Edward Jenner implemented the vaccination concept and used a less pathogenic cross-reactive virus from cows, CPXV, for smallpox protection (163). Over the centuries the virus used for smallpox vaccinations has evolved, differing now from CPXV (which nowadays very rarely infects cattle) and being called VACV (21). Humans were extensively vaccinated with VACV until smallpox was eradicated in Europe in 1953 (although minor individual outbreaks occurred) and globally in 1978 (74, 101). However, the vaccination coverage has consequently diminished, leaving the younger generations without protection against any OPVs, of which only CPXV is known to naturally exist in Europe (102). Indeed, European CPXV infections seem to be emerging (330). In addition to the eminence of CPXV and VACV in smallpox eradication and the implementation of vaccination methodology as a whole, VACV

has the honour of being the first virus vector carrying foreign functional genes into animals (236). Of all OPVs, this thesis concentrates on CPXV, although the term OPV is used when discussing cross-reactive antibodies.

The first descriptions of Borna disease (BD), then known as "hitzige Kopfkrankheit der Pferde", stem from equine practitioners in Germany in the 18th century (328). The disease was later named after a huge epidemic in cavalry horses in the city of Borna in Saxony at the end of the 19th century (92), and its viral aetiology was established as early as 1924 (350). In addition to certain parts of Germany, Austria, Liechtenstein and Switzerland have also constituted the classical endemic BD region where sheep, horses and rabbits have succumbed to the disease (92). In the 1990s, however, researchers started to find BDV elsewhere in the world as a result of mounting interest after the first reports of human BDV infections (267). Publications on BDV infection signs in multiple species, including the cat, dog and cow, and especially in neuropsychiatric human patients, have now accumulated (311). Many of the early PCR findings have later been shown or suspected to be caused by contaminations (248, 271), and there have been specificity concerns with some of the serological methods (271, 336, 343). Nevertheless, carefully verified human BDV infections have also occurred (83), but because the transmission between man and vertebrate animals has not been proven, BDV is strictly speaking a probable zoonosis. For simplicity, however, it is referred to as a zoonosis in this thesis.

Scientific knowledge of CPXV/OPV and BDV has proliferated greatly during this thesis work. Recent relevant literature has been included in the thesis, although the main hypotheses were formulated based on the situation at the beginning of the project. At that time, the first CPXV cases emerged in Finland and human BDV infection reports accumulated worldwide. Both pathogens were targeted, because they both were possible emerging zoonotic viruses, the presence of which could be investigated with similar research material.

Before this study, the occurrence of CPXV and BDV infections in Finnish fauna and humans was unknown. Therefore, the first aims were to establish adequate detection methods and screen samples from domestic and wild animals as well as humans to determine whether, where and in what species these viruses exist in Finland. In searching for and investigating wildlife reservoirs, wild mammal samples from two other Eurasian regions, Russian Buryatia in Siberia and northern Germany, were included, and an experimental BDV infection of the bank vole conducted.

2. LITERATURE REVIEW

2.1 DESCRIPTION OF THE PATHOGENS

2.1.1 Cowpox virus

Cowpox virus (CPXV) belongs to the family *Poxviridae*, subfamily *Chordopoxvirina*e and genus *Orthopoxvirus* (OPV) (54). This genus contains several cross-reactive and -protective species (Table 1). Other *Chordopoxvirus* genera, namely *Parapox-, Leporipox-, Capripox-, Avipox-, Suipox-, Yatapox-* and *Molluscipoxvirus,* include several other human- or animal-pathogenic and zoonotic viruses that do not cross-react with OPVs (74).

Table 1. *Approved virus species in the genus* ***Orthopoxvirus*** *(54), their host spectrum and distribution (74).*

Species, abbreviation	Reservoir hosts	Incidental hosts / Other infected hosts	Geographic range of natural infections	Zoonosis
Camelpox virus, CMLV	Camel	Nil	Africa, Asia	
Cowpox virus, CPXV	Bank vole (*Myodes glareolus*), field vole (*Microtus agrestis*), long-tailed field (wood) mouse (*Apodemus sylvaticus*); probably some other wild rodents	Cats, dogs, cattle, rodents, zoo animals, humans	Europe, Western Asia	X
Ectromelia virus, ECTV	House mouse (*Mus musculus*), other rodents?	Nil	Laboratories in Europe and USA	
Monkeypox virus, MPXV	Likely rodents	Nonhuman primates, zoo animals, prairie dogs, humans	Western and central Africa	X

Raccoonpox virus, RCNV	Raccoons	Nil	Eastern USA	
Taterapox virus, TATV	Gerbils	Nil	Western Africa	
Vaccinia virus, VACV	Unknown	Rabbits, cows, buffaloes, humans	Brazil, India (Laboratories worldwide)	X
Variola virus, VARV	Humans	Nil	Eradicated (formerly worldwide)	
Volepox virus, VPXV	California vole (*Microtus californicus*)	Nil	Western USA	

CPXV virions, 200 nm x 250–300 nm in size, are brick-shaped particles consisting of a surface membrane, a core including an enveloped nucleocapsid, and lateral bodies. During the replication cycle, extracellular, often enveloped, and intracellular, nonenveloped but mature virions are produced (Fig. 1). Extracellular virions initiate the infection, probably using macropinocytosis and apoptotic mimicry like the close relative, VACV (210). In the host cell, CPXV may be occluded within eosinophilic, type A inclusion bodies. Virions either remain inside the cell, being released as the cell lyses, or they bud through the host cell membranes as extracellular enveloped virions, consequently adhering to the cell surface and spreading to neighbouring cells or externalizing into the environment. (54, 218).

The CPXV genome is a nonsegmented, single linear double-stranded DNA molecule of 220 kb (kilo bases). The sequences are redundant terminally (54, 218, 287). The middle part of the genome is highly conserved and codes for proteins needed for the viral structure and replication. However, genes located nearer to the genome edges are slightly more heterogeneous coding for products needed for pathogenicity, such as immune modulators, and factors determining the host range and stimulating cell proliferation (74, 218, 287). OPV genomes can recombine when infecting the same cell (102, 218). Exceptionally for a DNA virus, it replicates in the cytoplasm. The replication cycle is complicated, including a strictly regulated cascade of gene expression from immediate early to late genes, long DNA concatemers, and a slow and complex assembly process (218).

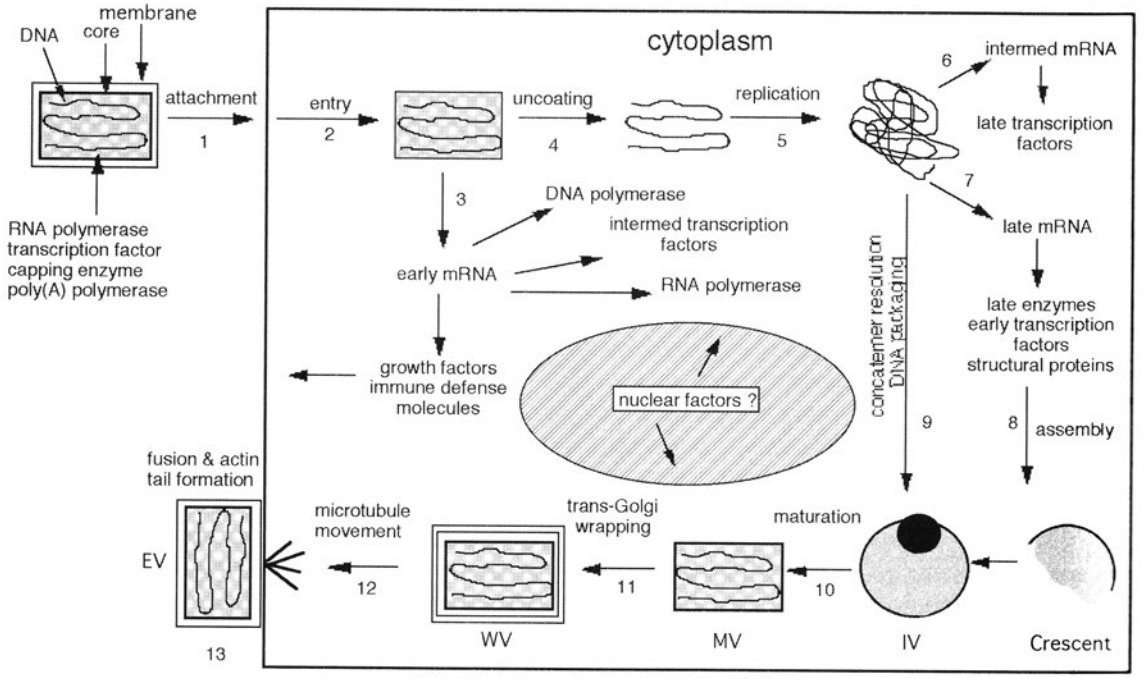

Figure 1. *Replication cycle of orthopoxviruses. A virion containing the DNA genome, enzymes and transcription factors attaches to a cell (1) and fuses with the cell membrane, releasing a core into the cytoplasm (2). The core synthesizes early mRNA that is translated into a variety of proteins, including factors for virulence, DNA replication and intermediate transcription (3). Uncoating occurs (4) and the DNA is replicated to form concatemeric intermediate molecules (5). Intermediate genes are transcribed and the mRNA translated to form late transcription factors (6). The late genes are transcribed, and the mRNA translated to form structural proteins, enzymes and early transcription factors (7). Assembly begins with the formation of membrane structures (8). The DNA intermediates are resolved into unit genomes and packaged in immature virions (IV) (9). Maturation proceeds to the formation of intracellular mature virions (MV) (10), which in the case of CPXV are partially occluded within inclusions. The MVs are wrapped by Golgi and endosomal cisternae (11) and the wrapped virions (WV) transported to the cell periphery (12). Fusion of the WVs with the plasma membrane results in the release of extracellular enveloped virions (EV) (12). An actin tail polymerizes beneath the EV (13) resulting in virus-induced, motile microvilli, on the tip of which most EVs adhere in the search for neighbouring cells. Reprinted from (218), with permission.*

2.1.2 Borna disease virus

Borna disease virus (BDV) was for a long time the sole member of the family *Bornaviridae* in the order *Mononegavirales* (81). Recently, however, its relatives, avian bornaviruses (ABV) have been detected in psittacine birds with proventricular dilatation disease (176).

BDV is an enveloped, 80–100 nm sized, noncytopathogenic, and strictly cell-associated virus causing slowly progressing, chronic infections of neurons *in vivo* but infecting several cell lines *in vitro* (76, 77, 192). BDV enters the target cell via receptor-mediated endocytosis (192) and leaves it by budding through the plasma membrane to the adjacent cell (most frequently) or intercellular substance (178, 195) (Fig. 2). The 8.9 kb single negative-stranded RNA genome replicates, as an exception among RNA viruses, in the nucleus (52, 53, 71, 82). By utilising splicing and alternate, overlapping reading frames, BDV uses its small genome effectively (Fig. 3) (53, 192). The genome encodes six proteins. The 38/39/40 kDa nucleoprotein (p40, N) is the most abundant of these, although when the infection becomes chronic, its molecular ratio to the 23/24 kDa polymerase cofactor phosphoprotein (p24, P; (309)), with which it forms complexes, decreases (332, 333). N and P are parts of the ribonucleoprotein (RNP) and they have an important role in the shuttling of the RNP between the nucleus and cytoplasm, and consequently in the control of the replication cycle (177, 192, 310). Phosphorylation of the P protein also contributes to efficient viral dissemination (276). In addition to these most abundant proteins, the BDV genome encodes protein p10 (X), matrix protein p16 (M), glycoprotein p57 (G, gp94 when glycosylated; further cleaved to gp43 and gp51), and RNA polymerase (L) (192, 310, 331). Polymerase activity is important in the adaptation of BDV to new hosts (5, 6), and X protein inhibits apoptosis, being essential for host survival (251). M and G proteins are essential structural proteins needed for the formation of BDV particles (242). BDV uniquely limits its genome amplification by trimming the genome at the 5′ terminus, which may favour noncytolytic viral persistence and the evasion of the antiviral host response (122, 278, 279). BDV infection produces an extremely low level of infectious virus particles per cell, although the cells express high BDV RNA and protein levels (76, 310).

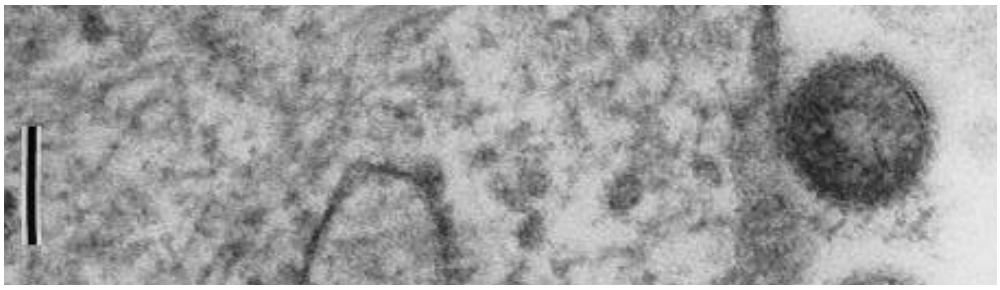

Figure 2. *The spiked Borna disease virus has just budded from a cell and become an extracellular particle. Electron micrograph; bar = 100 nm. Reprinted from (178), with permission.*

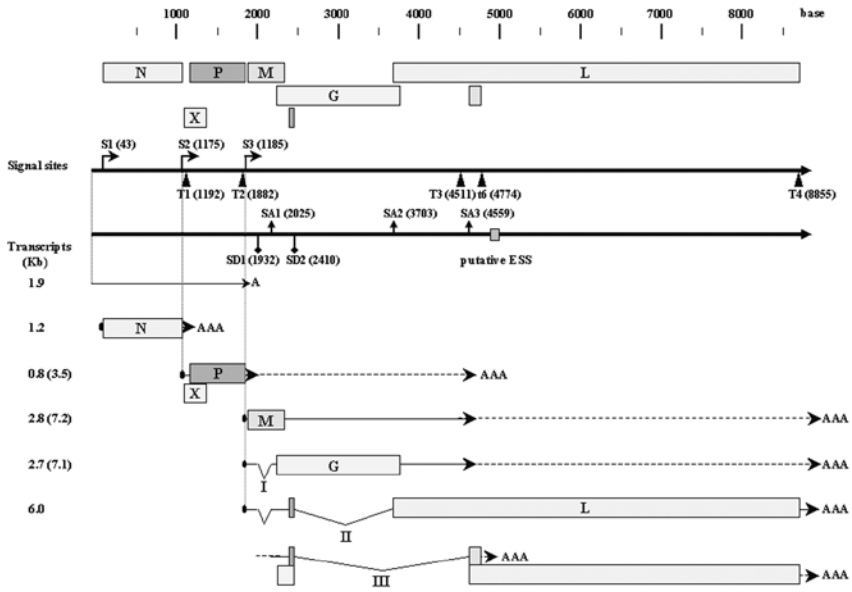

Figure 3. *Genomic organization and transcription map of BDV. N, nucleoprotein p38/p40N; X, protein X p10; P, phosphoprotein p24; M, matrix protein gp18; G, envelope glycoprotein gp94; L, polymerase protein p190; S1–S3, transcription initiation sites; T1–T4 and t6, polyadenylation/termination sites; SA1–SA3, splice acceptor sites, SD1 and SD2, splice donor sites; ESS, exon splicing suppressor. The positions of the sites are given for the antigenome in parentheses. Positions of the introns (I, II, III) are also indicated. Reprinted from (310), with permission.*

Very recently, ancient, endogenized bornavirus-like (EBL) sequences were detected in the genomes of humans and several other vertebrates (27, 155). The most commonly found EBL resembled the N gene (EBLN), and was the only EBL found in primates as well as in the guinea pig, squirrel, lamprey, and a few exotic animal species. In the genomes of the lemur, mouse, rat, microbat, wallaby, and a few fish species, however, either the L or M gene was detected in addition to or in spite of the N gene (27). Most EBLs contain numerous stop codons, thus remaining untranslated, but at least one human EBLN has been reported to interact with cellular proteins, indicating a natural function (155). The possible biological function of EBLs remains to be elucidated.

2.2 ECOLOGY AND EPIDEMIOLOGY

2.2.1 Stability of the viruses

OPVs are stable in the environment. Smallpox virus VARV is stable in scabs for 3–16 weeks, and ECTV in blood spots for up to 11 days (reviewed in (97)). VACV maintains its titre in vaccine vials at -20, 0, 4, or 25 °C for at least four days (188), and it is stable in numerous organic substances and water (1, 97). Few stability studies have been conducted on CPXV, but as it is biochemically equivalent to these other OPVs (218) it behaves similarly (199), and can be estimated to remain in the environment for days to months. The infectivity of free OPVs is readily destroyed at 56 °C and by ultraviolet light, as well as by low concentrations of formalin, lye, chlorine, iodine, peracetic acid, and hydrogen peroxide, but notably, cell-bound or dried viral particles withstand them for longer (199).

BDV remains stable for at least two years at -70 °C, one year at -20 °C, three but not four months at +4 °C, one week at +20 °C, two days at +37 °C, and 15 to 70 min at +56 °C (77, 195). It also tolerates alkaline and acidic pHs and drying quite well (77, 192, 195). BDV is sensitive to common disinfection methods including heat, organic solvents, detergents, chlorine, formaldehyde, a pH below 4 and ultraviolet light (77, 195).

2.2.2 Geographical distribution

Natural CPXV or closely related OPV infections exist in many Eurasian countries (Fig. 4). In most of them, it has been possible to carefully verify the existence of CPXV with virological and molecular methods, but in some areas the distribution map is based on serology (184) or the molecular methods have not reliably differentiated the infecting virus species from other OPVs (61).

Signs of BDV infection, including antibodies, antigen, RNA and/or virus itself, have been reported from all continents except for South America (Fig. 4). The highest clinical incidence in animals and the verified classical Borna disease cases, however, are restricted to central Europe (90, 299). As several PCR amplicons retrieved from animals and humans outside the classical endemic central-European area resemble those of control strains, many PCR results have been suspected to be caused by contaminations (89, 91). Furthermore, some epidemiological studies have been conducted based on a triple enzyme-linked immunosorbent assay (ELISA) method, which was published without negative controls, but is claimed to detect circulating BDV immunocomplexes (CIC), antigen, and antibodies (47). Its results have not been reproducible or confirmable with other methods (80, 343). However, these

discrepancies cannot explain the detected BDV antigens or antibodies with other, reproducible methods, e.g. immunostaining and IFA. Thus, although it seems probable that not all BDV reports are reliable, the virus or its close relative is widely distributed. Whether some of the findings are in fact caused by ABV, EBLs or as yet unknown bornaviruses remains to be shown.

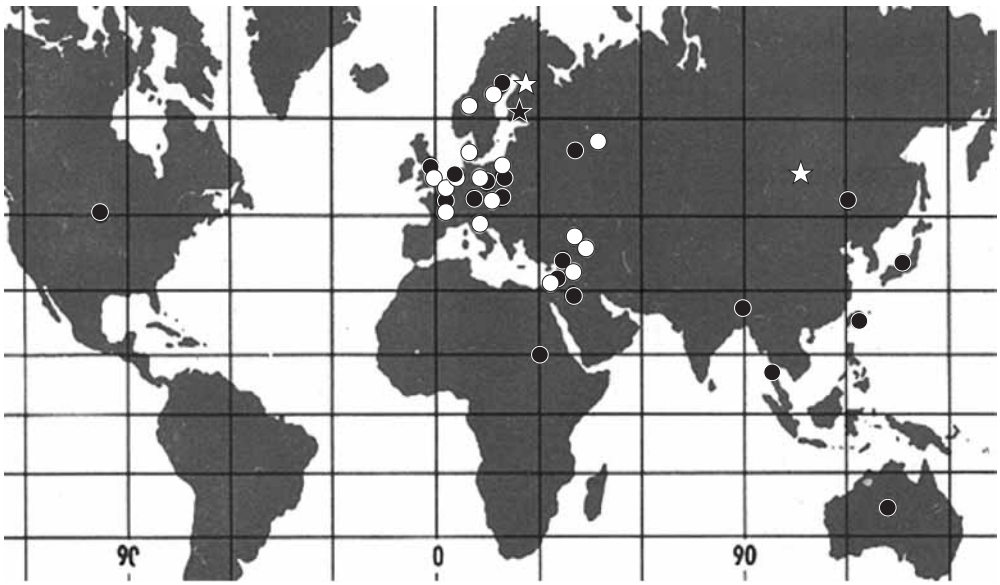

Figure 4. *Distribution of natural CPXV or closely related OPV infections (❍) (51, 61, 114, 151, 184, 203, 214, 219, 223, 305, 315, 322, 346) and reported signs of BDV infection (●) (13, 14, 42, 65, 110, 124, 148, 158, 196, 201, 212, 221, 257, 267, 269, 285, 338, 347, 351). Countries such as Italy and the Czech Republic, the BDV findings of which are solely based on the controversial triple ELISA method, have been omitted (80, 245, 256). New locations for OPV and BDV identified in this study are marked with ☆ and ★, respectively (174, 175, 241).*

2.2.3 Species diversity

In contrast to most other poxviruses, which have only one host, CPXV can infect numerous vertebrate species. In addition to the cat, which is the most commonly detected victim of CPXV, infections have so far been detected in three primate (humans, the Barbary macaque, marmoset), eleven other feline (the cheetah, lynx, lion, leopard, black panther, ocelot, jaguar, puma, far eastern cat, Bengal cat, jaguarundi), six other carnivorous (the dog, fox, arctic fox, bandeed mongoose, stone marten, bearcat), four cloven-hooded (the wild boar, okapi, cow, lama), four odd-toed ungulate (the horse, black rhinoceros, white rhinoceros, tapir), fourteen rodent (such as the beaver, bank vole, yellow-necked mouse, ground squirrel and Patagonian mara), and three other animal species (the Asian and African elephant, anteater, common

shrew) (reviewed in (98, 219)). Despite the wide host range, only a few species are capable of effectively transmitting the virus further. These principal hosts maintain the virus in the population, and are considered to include at least the bank vole (*Myodes glareolus*), field vole (*Microtus agrestis*), and long-tailed field / wood mouse (*Apodemus sylvaticus*) (63, 292). In addition, numerous other rodent species may become infected, but their role in viral epidemiology is unclear (96, 98, 202, 204, 317, 318). Hosts predominantly not transmitting CPXV further are termed incidental hosts, and even the cow belongs to this group. Therefore, "cowpox" is a misnomer and should be updated to "rodentpox" according to the principal reservoir hosts.

BDV has long been known to infect and cause disease in horses (351). Later, natural infections with BDV or a BDV-like agent have also been verified in zoo animals (the monkey, sloth, llama, alpaca, pygmy hippopotamus; (160) and reviewed in (90, 158)), the goat, deer (reviewed in (92)), sheep (212), rabbit (211), cat (33, 164, 196, 229, 257, but 226), cow (58), human (83), dog (338), lynx (84), and recently, the shrew (150). Evidence also exists of infections in the ostrich (200), fox (78), mallard and jackdaw (36), macaque (127), and raccoon (126). In addition, this neurotropic virus experimentally infects the tree shrew, rhesus monkey, chicken, rat, mouse, hamster, Mongolian gerbil, and guinea pig (reviewed in (250)). Despite the wide possible host range, the incidence of BD in species other than horses and sheep appears low (90).

As sporadic and relatively poorly-known diseases, both cowpox and Borna disease commonly seem to escape diagnosis.

2.2.4 Phylogeny and molecular epidemiology

Most known OPV genes are highly conserved within the genus: particularly those located in the central genome commonly exhibit a nucleotide identity higher than 90% (74, 88, 193). However, if genes that are more variable are analysed, all other species isolates in this genus cluster together and separately from other OPV species, but CPXV isolates often scatter in several phylogenetical clades or restriction pattern groups (Fig. 5) (120, 193, 214). Some isolates and genes resemble more the VACVs and others close in the VARV/CMLV group (120, 131, 146, 193). There is evidence of recombination, which might partially explain this phenomenon (120). Many scientists have even argued that different CPXV isolates should be separated into diverse species instead of referring to all of them as CPXV (120, 193). The clustering of CPXV isolates shows little correlation temporally, geographically or according to the species of origin (172, 193, 213), although evidence of slight geographical clustering in Scandinavia has recently emerged (132).

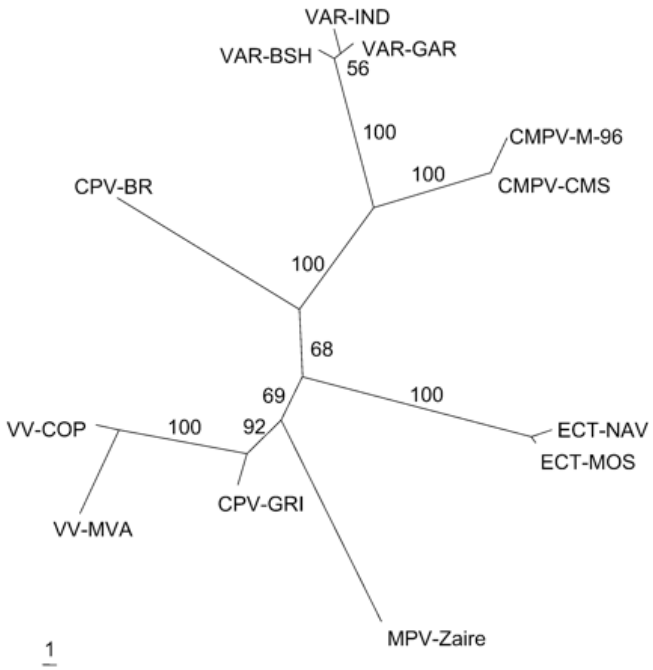

Figure 5. *Phylogenetic tree of 12 OPVs obtained by the maximum-likelihood method using amino acid sequences of 12 OPV proteins (VV-COP C6L, C7L, N1L, K2L, F2L, F4L, F6L, F8L, A56R, B1R, B5R, B15R) encoded in the terminal regions of the genomes. The maximum-likelihood tree is shown in an unrooted format. The bootstrap values from 1000 replica samplings and the divergence scale (substitutions per site) are indicated. Reprinted from (120), with purchased permission.*

The genomic sequence of BDV — then representing all known bornaviruses — was long thought to be extremely conserved, as the nucleotide sequences from isolates or PCR amplicons were more than 95% identical (39, 90, 149, 277, 299). As BDV has an RNA genome and lacks the proof-reading activity of its polymerase, it has also proven surprisingly stable in persistently infected cell cultures (107). A strict selection pressure, probably related to a complicated transmission chain including several species, such as with alpha- and rabies viruses (152, 334), has been assumed to provide constraints to genomic variation (90). More recently, however, one strain from an Austrian region where BD had not previously been diagnosed, has been detected with only 85% identity to the previous variants (228). This finding indicates that additional BDV variants with distinct features may be present, but probably escape detection by the common PCR methods. Even the most similar central European BDV strains present with minor differences according to their geographic, but not host species, origin (179). Recently, a BDV-related new virus species, ABV, was found with modern, virus family-specific hybridization methods. ABV isolates possess a nucleotide identity of only 60% to the known BDV strains (Fig. 6) (176). Surprisingly, the very recently observed, 40-million-year-old bornavirus-like genes in vertebrate genomes have also

retained their similarity to current BDV strains well: the EBL sequences are 40% identical to the current viruses at the amino acid level (155). Thus, bornaviruses overall seem not to evolve according to a molecular clock similarly to other RNA viruses, but are highly conserved (155).

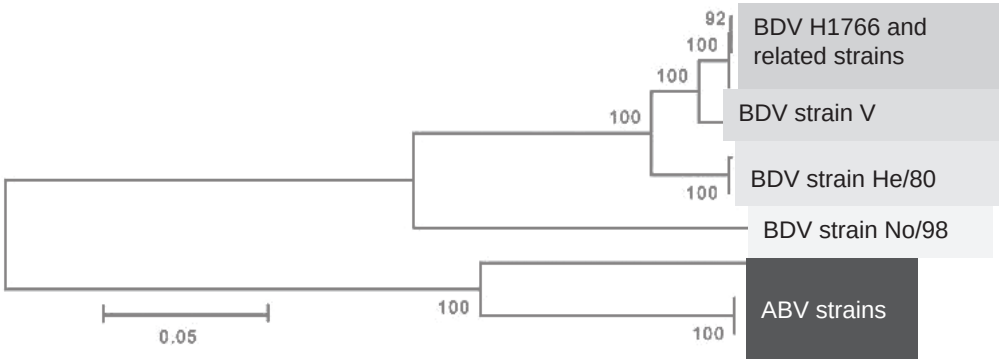

Figure 6. *Phylogenetic tree of currently known bornaviruses based on 5.5 kb-long nucleotide sequences and the neighbour-joining method. Adapted from (153), with permission. BDV, Borna disease virus; ABV, avian bornavirus*

2.2.5 Other epidemiological facts

Although CPXV and BDV both infect numerous animal species, they are not very contagious within a domestic animal species, and apparently not from domestic animals to humans. Human CPXV infections most commonly arise from cats or rats (23, 162), but direct transmission from the cheetah, cow and Asian elephant has also occurred (reviewed in (98)). The infection source, however, often remains unresolved (23). Despite being the most common infection route, CPXV is not easily transmitted from cats to humans (31). The risk of human infection is probably higher if the cat's condition is not diagnosed and proper protective measures undertaken. Human-to-human CPXV transmission has not been proven (23), and symptomatic cat-to-cat transmission of natural cowpox is also rare (31). This has been verified in experimentally infected cats: the sentinel contact cats seroconvert but remain asymptomatic (32).

Similarly to cowpox in cats, cases of Borna disease in horses and sheep are also mainly sporadic, and transmission within or between the domestic species such as sheep, horses, and cattle rarely if ever occurs (reviewed by (299)). However, the stable mates of BD animals frequently have or gain antibodies, indicating a common transmission source (299, 321). Although the disease seems to appear in cats of the same regions in the course of time (339), contact with cats in the household or fighting with other cats are not risk factors for feline Borna disease (33). On the contrary, roaming free in a rural environment or hunting rodents increase a cat's BD risk seven-fold. Likewise, the

majority (22 of 25, 80%) of the cats with verified cowpox were found to originate from rural or suburban environments, and at least 76% of them evidently hunted rodents (31). This type of epidemiology naturally stems from the fact that the principal hosts for CPXV are wild rodents, in which CPXV mainly circulates, and only occasionally infects the incidental hosts such as cats and humans (63). As described above, the same is indicated to apply to BDV, as also further evidenced by several other epidemiological data sets suggesting a reservoir in small wild mammals (90, 299). First of all, BDV prevalence is higher on farms lacking proper rodent control and hygiene. Secondly, focal BDV epidemics are observed independently of the region and species at 2- to 5-year intervals (90, 92), which could be related to fluctuation in wild rodent numbers. Thirdly, BDV is transmitted horizontally in laboratory rats via urine (273) and probably vertically (reviewed by (90)), and fourthly, BDV strains cluster geographically rather than according to the species or year of isolation, suggesting that transmission within a domestic species is uncommon (179).

There is, however, one major difference in the epidemiology of BD and cowpox: the incidence of BD in horses and sheep peaks in March to June and in cats between December and March, although cases in all these species occur year-round (90, 92, 196). Quite the contrary, the striking peak in feline and human cowpox number appears in late summer and autumn (July to October), thus better reflecting the rodent numbers, which peak in the same season (172, 244, 325). The temporal appearance of cases is strictly associated with the incubation period. For CPXV it is short, from 3 days to 3 weeks, so the cases are seen almost simultaneously with the peaks in rodent numbers. The natural incubation period for BDV is much longer: based on practical experience it is 2 to 3 months (90, 160), but probably even up to 143 days in horses (207) (see also "2.3.2 BDV infections"). If this holds true, horses and sheep should become infected between November and April, at the time when the few living rodents seek shelter inside or start mating.

Despite the above-mentioned numerous epidemiological facts and the recent finding of BDV infection in shrews (150, 252), some scientists object to the "BDV reservoir theory". They support their argument with the extremely high BDV prevalence (30-100% (238)) in humans and horses so enabling direct transmission between individuals. These prevalences are, nevertheless, based on the controversially discussed triple ELISA method ((41, 47, 343); see " 2.2.2 Geographical distribution"), and other research groups utilising other methods have suggested much lower prevalences (summarized by (158)).

Both cowpox and Borna disease are sporadic maladies. Altogether, around 100 reported human and tens of animal cowpox cases have occurred. Moreover, the incidence of Borna disease even in the best known hosts, horse and sheep in the classical endemic region, has declined from the large outbreaks affecting thousands of patients in the 19th century to fewer than 100 cases in each species a year (reviewed in (299)).

2.3 DISEASES CAUSED BY COWPOX AND BORNA DISEASE VIRUSES

2.3.1 Natural CPXV infections

Clinical cowpox is most commonly diagnosed in humans and cats, although several other species may succumb to the disease (Fig. 7, Tables 2 and 3). CPXV is considered to enter an animal host through the skin, mucosal membranes or respiratory tract. After initial infection, the virus moves to regional lymph nodes and spreads again from there, resulting in primary viraemia and multiplication in reticuloendothelial cells. After the multiplication and paralleling the incubation period of usually 7–14 (3–21) days, secondary viraemia combined with rash, constitutional symptoms and virus excretion ensues (32, 74). In humans, skin abrasions are the most common portal of entry, typically resulting in lesions either on the fingers, hands or face (23). The normal clinical course of infection in immune competent individuals of most species, and also in humans, includes inflamed and painful macular, papular, vesicular, pustular, ulcerative and eschar stages of 0.5–2 cm of size followed by healing in 3–12 weeks (23, 31, 56). Constitutional symptoms are very common in humans but less commonly observed in cats (23, 31, 117). On the contrary, secondary lesions are much more common in feline than human patients, although they both usually present with few (one to two) primary lesions (23, 31). Localized skin lesions show necrotizing dermatitis and folliculitis characterized by hypertrophy and basal cell proliferation together with inflammatory infiltration; A-type inclusion bodies are pathognomonic, although rarely observed in human lesions (23, 74, 172).

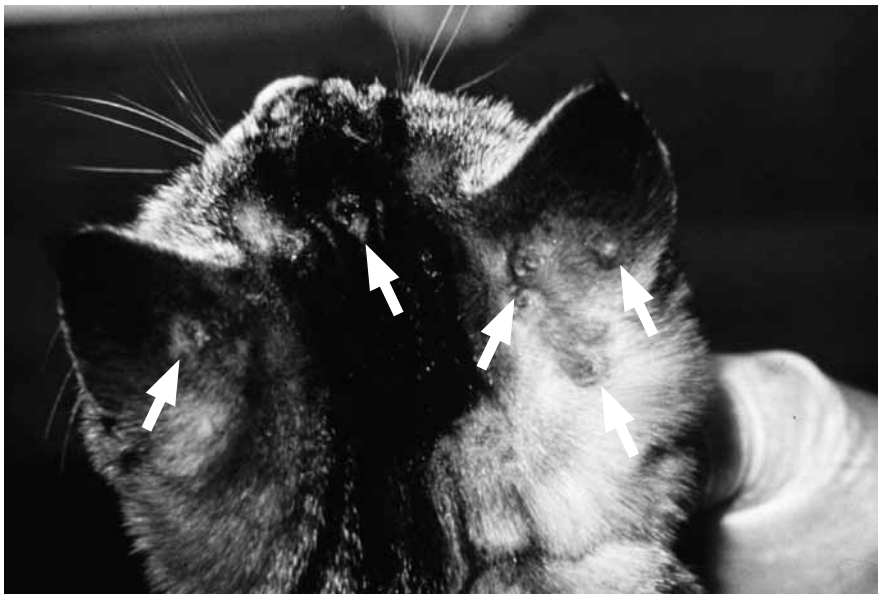

Figure 7. *Healing cowpox lesions on a cat's head. Photo: Malcolm Bennett, with permission.*

OPVs, especially CPXV with its large genome, encode and express a large number of intracellular and secreted proteins that modify the host responses to the OPV infection (74). These immunomodulatory proteins, for example, inhibit apoptosis and immune functions of dendritic cells, and bind to host cytokines, components of the complement system, chemokines, interferons, and their receptors (74, 133).

In some individuals, especially those who are immunocompromised or immunomodified, cowpox can be severe or even lethal (31, 72, 117, 151). For example, in human atopic dermatitis, the unusual abundance of Th2 cytokines downregulates the antiviral innate immunity of keratinocytes, allowing increased replication of OPVs (74). Examples of different clinical pictures are presented in Tables 2 and 3. Differential diagnoses in cats include other severe ulcerating and necrotizing dermatitis, e.g. eosinophilic granuloma, neoplasia, pyoderma, mycosis, pemphigus, and herpes- or calicivirus infection (172, 244). In humans, cowpox may resemble infections with herpesviruses (especially varicella-zoster) or parapoxviruses from ruminants, i.e. farmyard poxes, or anthrax, cat scratch disease, i.e. bartonellosis, rickettsioses, or ulceroglandular tularaemia.

Table 2. *Clinical cowpox / orthopoxvirus[a] infection manifestations in animals.*

Species	Main clinical findings	Outcome / recovery time	Specific	Zoonotic transmission	Reference
Domestic cat	Single primary lesion (sometimes resembling a chronic ulcer), mainly on the head, neck or forelimb. Multiple secondary skin lesions 4 – 16 days after the appearance of the primary lesion. Rarely anorexia, pyrexia, depression and tachypnoea associated with the onset of secondary lesions.	2–12 weeks (n=37) or death from cowpox (n=1) or because of renal failure (n=1) or euthanasia (n=9)	One littermate of a diseased cat developed severe disease when the lesion of the index case had almost healed.	Yes (2 cats)	(31, 243, 313, 325)
	Chronic wound or few scabbed lesions on the head; later generalisation.	4 and 10 weeks; 1 cat was euthanized.	In all 3 cats, generalisation occurred after treatment with corticosteroids.	No	(18, 243, 335)
	Multiple skin lesions, conjunctivitis or blepharitis; transient neurological signs	>5 weeks / euthanasia	3 cats, 2 of them kittens	No	(111)
	Necrotizing, ulcerative, generalised dermatitis	8 weeks	Probably transmission to a dog of the same household (see below)	Yes	(326)
	Vesicular dermatitis, extensive cellulitis, severe buccal ulceration	Death	Feline leukemia virus antigen positive	No	(31)
	Necrotizing, ulcerative, generalised dermatitis and glossitis	Euthanasia on day 17 after onset		Yes	(349)
	Necrotizing, rapidly progressive facial dermatitis, pneumonia	Euthanasia	Systemic infection verified *post mortem*	Probably	(146)

	Necrotizing pneumonia +/- generalised ulcerative dermatitis (2 cases)	Euthanasia		1: No 2: Yes	(151, 198)
Rat	Skin lesions on extremities, mouth and nose	May be fatal		Yes	(24, 55)
	Respiratory infection	Death in a few days	CPXV detected in the owner	Yes	(24)
Big felines: lion, panther, puma, cheetah, ocelot, jaguar, leopard	Severe bronchopneumonia or either local benign or generalised pustular dermatitis. Mixed manifestations also known.	Local dermatitis: yes, bronchopneumonia or mixed form: death			Reviewed in (219)
Dog	Crusted wound in lip	2 weeks	Diseased 2 weeks after a cat of the same household developed dermatitis (see above)	No	(326)
	Nonpruritic ulcerated / nodular lesion in muzzle (2 cases)	Yes	At the site of a rodent bite (1 dog)	No	(291, 327)
Cow	Fever, local haemorrhagic pock lesions (udder, teats, scrotum)	Yes, 7 weeks	Incubation time 2 – 7 days, newly calved cows less severely affected	Possibly	(114) and reviewed in (219)
Horse	Fever; lymphadenitis; vesicles and pustules most commonly in the hollow of pastern but anywhere on the body	3 weeks	Referred to as horse-pox, transmittable to cows and man closely resembling VACV vaccination, not verified with modern methods	Yes	(197)
	Papuloulcerative dermatitis on the body, tongue and mucosal membranes	< 1 week	Associate horses remained healthy	No	(243)

	Severe ulcerative stomatitis, oesophagitis, and gastritis in a foal (colitis, polyarthritis, nephritis)	Euthanasia	Combination with premature birth, lack of colostrum and streptococcal septicaemia. The dam was healthy.	No	(94)
Giant anteater	Generalised disease with pulmonary and mucocutaneous manifestations	Death in 3–4 weeks			Reviewed in (219)
Okapi	Local or generalised dermatitis	Local: 5–6 weeks; Generalised: death			Reviewed in (219)
Asian elephant	Apathy, malaise, vesicles and later ulcers up to 15 cm around the mouth and trunk	Euthanasia	Not vaccinated; transmission probably from rats	Yes	(143, 182)

[a] The microbiological diagnosis was not set at the species level in all described cases.

Table 3. *Different cowpox / orthopox[a] virus infection manifestations in humans.*

Main clinical findings	Outcome / recovery time	Specific	Zoonotic transmission	Reference
1–3 lesions on the fingers, hand or head (51 cases); or elsewhere (16 cases); or localization not reported (2 cases). Lesions up to 3 cm in diameter develop through macular, papular, and vesicular stages in 7–12 days, later pale blue-purple and increasingly haemorrhagic, umbilicated vesicle, pustule and ulcer by the end of second week. Healing by scarring through hard, deep-seated, black eschar. Most patients also have pyrexia, malaise, lethargy and occasionally vomiting and sore throat.	Mainly 6–8 weeks (3–12)		Yes: bovine, feline, rodent, elephant	(23, 24, 61, 67, 68, 104, 123, 143, 198, 243, 281, 282, 304, 313, 322, 342, 344, 348, 349)
6-9 lesions on arm	6–12 weeks	Born 1989 and 1991[b]	Yes, cat	(103, 243)
Large single lesion(s) (up to 4 cm), malaise, painful lymphadenopathy (2 cases)	Yes	1: Woman, born 1984 2: Veterinary student, born 1990	1: Probably, cat 2:Probably, diverse animal contacts	(68, 116).
Several papular and later hemorrhagic ulcerative lesions on the forehead, tongue, neck, shoulders and thumb, general malaise, headache, fever, lymphadenitis	Several weeks	Born 1993	Rat	(24)
Inflamed lesion on the head/neck; later satellite lesions			Probably, cat	(137, 300)
(Severe) conjunctivitis (5 cases), keratitis (1 case)	Yes, apart from one conjunctivitis patient. Keratitis in 9 months.		In one case: rat	(23, 24)
Necrotizing cellulitis of the nasal tip and vestibule, lymphadenitis, fever	6 months		Probably, dog	(189)

Extensive necrotic malignancy of the cervical soft tissues (2 cases)	Cellulitis in 3 weeks, necrotizing lymphadenitis in >2 years	1: Born 1993 2: Born 1990	1: Unknown (cat?) 2: Rat	(24, 235)
Pneumonia	Several weeks	Born 1985	Rat	(24)
Generalised dermatitis (4 cases)	All: yes. 2 cases: 4 weeks	History of atopic eczema (n=3) or hay fever	2 cases: Probably, rat and cat	(40) Reviewed by (23) (154)
Confluent, haemorrhagic vesicopustular dermatitis on the whole body	Death from cardiac arrest caused by pulmonary embolism	Severely immunosuppressed	Yes, cat	(72)

[a] The microbiological diagnosis was not set at the species level in all described cases.
[b] Birth years presented either as reported in the article or estimated on the basis of the reported age combined to the date of publication.

2.3.2 BDV infections

BDV experimentally infects animals via various routes: intranasally, intracerebrally, intramuscularly, subcutaneously, intradermally, and less efficiently intravenously, but not intragastrically (60). Natural transmission routes are unproven. Nonetheless, successful experimental intranasal infection of rats (60, 216, 301), mice, sheep, and horses (299) and the presence of BDV antigen or -RNA in the olfactory epithelium, nerves and bulb of naturally infected horses strongly point towards a nasal or olfactory route, although trigeminal and pharyngeal nerve routes cannot be excluded ((38) reviewed in (262, 301)). Infection most probably occurs via excreta: experimentally chronically infected rats excrete BDV, for instance, in tears, saliva and urine, and can infect other rats in close contact (216, 273, 303). In a few naturally infected horses and sheep, BDV RNA is found in salival, nasal and conjunctival fluids, but surprisingly, infectious virus is nonexistent in asymptomatic animals (148, 186, 260, 321) and rarely exists in symptomatic horses (148), further indicating possible transmission from a reservoir (186, 260, 321). In addition to horizontal transmission, BDV has transmitted vertically in experimentally infected mice (231), probably rats (reviewed by (90)), and possibly from a pregnant mare to her fetus (125).

BDV spreads intra-axonally from the inoculation site into the central nervous system (CNS) (60). When in the rat CNS, the virus rapidly spreads and can be demonstrated in all cortical and brain stem areas at day 10 p.i. (301). Naturally infected horse brains are also widely BDV positive (38), which may be followed by intra-axonal centrifugal spread to peripheral nerves, and occasionally, to other than neuronal cell types (158, 181, 186).

In cats experimentally infected by intracerebral (i.c.) inoculation, the first symptoms may arise on day 14 p.i., but it may take 75 days to detect them or a cat may remain asymptomatic for at least 6 months (164). Ponies experimentally infected by i.c. inoculation had an incubation time of 15–26 days (171), and older studies have proposed incubation periods of 24–143 days in horses, 32–85 days in sheep, 18 days to months in rabbits, 20–390 days in guinea pigs, 20–90 days in adult rats, and 36 days in hens (207). However, experienced clinicians estimate an average incubation time of 2–3 months in horses (90), and recent data indicate a minimum of 2 months in alpaca (160). Persistent subclinical infections, resulting either from a very long incubation period or the low-pathogenic character of the BDV infection or both, are common. Indeed, during a follow-up of 1–2 years in naturally infected animals, only a proportion of the antibody-positive individuals (20% of horses, up to 40% of sheep) developed symptoms ((148, 321) and the review by (299)). In naturally infected, diseased animals, BDV infection manifests as a peracute, acute or subacute disease with nonpurulent meningoencephalitis, although milder manifestations are also seen (Table 4; (34)). Different combinations of simultaneous or successive neurobehavioural alterations are noticed (38, 119, 158, 212, 259). Paralysis is common, and in the

classical BD form, death occurs in 60–80% of the animals by 5 weeks after the onset of symptoms. Spontaneous recovery is possible, but is often accompanied by altered behaviour for the rest of the animal's life, and occasionally leads to the recurrence of severe encephalitis (119, 158, 261).

In experimental murine infections, the disease onset correlates with viral expression in the lower brain stem and cerebellum, although BDV is mainly found in the cerebral cortex and hippocampus (332). In infected rats, BDV has several pharmacological effects in the brain, such as upregulation or activation of certain kinase pathways, transcription factors and genes resulting in either dopaminergic, and finally fatal meningoencephalitic disease in immunocompetent adults, or persistent, global development disorder manifesting with hyperactivity and learning disturbances in those who are neonatally infected (250, 294, 295). Rat infection with one particular strain leads to the development of obesity syndrome, which has been hypothesised to result from neuroendocrine dysregulation by BDV (145). Furthermore, BDV seems to impair the functions of nerve cells through interference with the protein kinase C-dependent signalling by the P protein, by blocking stimulus-induced synaptic activity, one form of neuronal plasticity thought to be important for learning and memory (324). Moreover, BDV effectively inhibits activation of the intracellular pathogen recognition receptor RIG-I, which leads to protection from the antiviral actions of innate immunity (122). Recent studies have revealed additional mechanisms by which BDV interacts with host cells and affects innate immunity (254).

Interestingly, BDV pathology is partially related to the T cell-mediated immune response (128, 301). CD4+ (helper) T cells are the most abundant and predominate in the perivascular inflammatory cuffs, whereas cytotoxic CD8+ T cells are present in the brain parenchyma, especially in the vicinity of possible degenerating neurons, indicating a role in the pathogenesis of BD (301). Indeed, athymic, splenectomised or otherwise immunocompromised rhesus monkeys, mice or rats do not show BD or acute inflammatory reaction, while the untreated animals succumb to severe, even lethal BD ((128, 222, 302) reviewed in (301)). This has been attributed to the presence or absence of the CD8+ T cells and a delayed type hypersensitivity reaction, although sole production of certain proinflammatory cytokines, such as γ-interferon, may also contribute, as recently shown in feline infections (301, 340).

Table 4. *Manifestations of Borna disease in natural host animal species.*

Species	Clinical manifestation	Pathological manifestation	References
Horse	Begins with excitability or depression; continues with swallowing difficulties, ataxia, imbalance, decreased sensory, proprioceptive and reflex functions; ends with severe excitability, aggressiveness, or lethargy, and circling, paresis, paralysis, somnolence, stupor and coma.	Meningoencephalitis (myelitis)	(38, 92, 119, 158, 259, 306)
	Ataxia, narcolepsy, behavioural changes, apathy, colic, abnormal movements	Not analysed	(34, 44)
Sheep	Altered behaviour, progressing ataxia, swallowing difficulties, wall-pressing, somnolence, dyskinesia (seizures)	Encephalitis (meningitis, myelitis)	(212), reviewed in (158, 194)
Cow	Anorexia, anxiety, ataxia, paresis, circling, paralysis	Encephalitis	(58, 230)
Alpaca	Lack of sexual desire, convulsions, prostration, death	Meningoencephalitis	(160)
Rabbit	Anorexia, apathy, somnolence, gait disorders, paralysis (seizures)	Meningoencephalitis, myelitis	(211)
Dog	Salivation, mydriasis, circling, coma	Meningoencephalitis	(232, 338)
Cat	Mental and behavioural changes, hind leg ataxia, paresis, anorexia, hyperaesthesia (seizures)	Meningoence-phalomyelitis	(164, 196, 229)
Ostrich	Incoordination, paresis	No specific lesions	(200, 201)

2.3.3 Human Borna disease?

As BDV infects a wide variety of animal species with a predilection for the limbic system, and occasionally induces persistent emotional, cognitive and behavioural alterations in experimental animals, including primates and their ancestors (297, 302), the question has arisen of whether the virus could also be a human pathogen and cause neuropsychiatric disorders. The first evidence of human infections was, indeed, reported in 1985: 0.43–12.5% of patients with diverse psychiatric disorders had BDV-antibodies in the serum whereas the prevalence in the control group was <0.5% (11, 267). The seropositive patients were diagnosed to have a primary major depressive disorder. The majority of the later studies have also revealed higher BDV marker prevalence in some patient groups than in controls, i.e. in unspecified psychiatric in-

patients, neurotic, personality adjustment and mood disorder patients, schizophrenics and patients with chronic fatigue syndrome (summarized by (59, 62, 246, 308)). However, signs of infection are not present in all patient groups, and control or comparison groups also sometimes harbour signs of BDV infection. Furthermore, despite the association of detected BDV markers with some psychiatric diseases in numerous studies, conclusions on the causal relationships are impossible to draw. The situation is even more complicated by the facts that no consensus exists on the method which should be used for BDV diagnosis (41, 62, 271, 336, 343), and that based on the sequence identity, many PCR results and even virus isolation from human peripheral blood cells have been suspected to have arisen from contamination by a control strain (89, 91, 190, 227, 248, 271, 284).

Despite the controversies, there are researchers who consider that the existence of human Borna disease has been demonstrated and it is common (45-48, 194, 238, 239, 280). Especially controversial, however, are findings based on the ELISA method detecting BDV antibodies, antigen and CICs ((47, 343); see "2.2.2 Geographical distribution"). Moreover, the lack of specificity of other serological methods has been surprisingly strongly suspected if compared to the diagnostic methods for other viral infections (271, 336). Nonetheless, a few human BDV infections have been carefully verified with a combination of methods including reproducible and controlled antigen detection by immunohistology, as well as RNA detection by *in situ* hybridization and RT PCR in the brain tissue of patients suffering from hippocampal sclerosis (83). Furthermore, the recent EBLN findings in primate genomes prove that our ancestors were infected with a bornavirus (27, 155). These facts convincingly verify that BDV can indeed infect humans. However, the frequency of human infections, the role of EBLNs in human disease, and the existence of human Borna disease are still questionable (27, 90, 159, 191, 298, 299).

2.4 INFECTION KINETICS AND DIAGNOSIS OF CPXV AND BDV INFECTIONS

2.4.1 Immunity and detection of serological response

In acute OPV infection, cytokines and cytotoxic CD8+ T cells are crucial to control the disease, but for complete virus clearance and recovery, B cell functions and therefore antibodies are necessary (64). In the mouse model, antibodies also prevent virus from forming pock lesions on the skin and inhibit transmission (64). Neutralizing antibodies are assumed to prevent a reinfection in humans, and as cross-reactive antibodies after infection with one OPV persist even for decades, the relevance of smallpox vaccination in inhibiting all OPV infections in humans is easily deduced (74, 173). After experimental feline infection, haemagglutination-inhibiting antibodies are

present on days 6–16 p.i. and thereafter, corresponding to approximately one week after the onset of skin lesions, but a high titre of neutralizing antibodies seems to need a few more days to develop (29, 32, 111). Antibodies last no less than 6–10 months in cats (29, 111). Rodents experimentally infected intradermally and subcutaneously become seropositive 9–12 days p.i., but the majority of those infected with a low dose or via the oronasal route remain antibody-negative (30).

In contrast to acute OPV infections, antibodies do not seem to play a protective role in persistent BDV infections: even 60–70% of naturally infected animals do not mount an antibody response at all, and if they do, the titre is low and neutralizing antibodies are detected late, if ever (119, 135, 164, 271, 321). Furthermore, seroreversion may be possible (142). Although the relevance of neutralizing antibodies is difficult to determine in natural infections, prophylactically administered neutralizing antibodies against the major glycoprotein prevent infection and encephalitis in experimentally infected rats (109). If neutralizing antibodies are administered simultaneously with the virus, they can still inhibit the viral spread to peripheral tissues and prevent virus excretion and transmission, although not the persistent BDV infection itself (303). Immunosuppressed rats (which present with subclinical BD) are competent to mount a humoral but not a cellular immune response (303).

Horses and cats experimentally BDV-infected through i.c. inoculation seroconvert one month p.i. (164, 171). Depending on the viral strain, cats have a humoral response to the P protein or both N and P proteins (164). This difference may be related to the stage of viral persistence: the N:P ratio decreases as the infection becomes chronic (186, 333). Overall, the serological response is stronger in experimentally than in naturally infected animals although not constantly detected in either animal group (147, 164, 216, 268).

BDV antigen has been claimed to be present in the peripheral blood and to commonly bind to BDV-specific antibodies, so forming circulatory immunocomplexes, CICs (47). CICs have been detected with a reverse-type sandwich ELISA by several, associated research groups reporting high BDV prevalences among humans, horses and cats (41), although other researchers have been unable to reproduce them (343). Lower prevalences are found with more conventional methods, of which the immunofluorescence assay (IFA) is the most commonly used (267, 271). Immunoblotting, immune precipitation, the electrochemiluminescence immunoassay as well as different versions of the ELISA method are also used for BDV antibody detection from serum and cerebrospinal fluid (reviewed in (271)).

Measurement of the avidity, i.e. functional affinity, of antibodies has long been used in the timing of primary infections: a high proportion of strongly-bound, urea-resistant antibodies indicates long-term (weeks to years old) adaptive immunity, whereas in acute infection the proportion is low (140). Within the debated field of

human BDV infections, high avidity, or especially the lack of it, has also been utilised as an indicator of the specificity of the antibody response (8, 37). Although most BDV antibody findings in humans have been of low avidity (8), high-avid serological responses also exist (37).

As CPXV infections in incidental hosts cause clearly detectable dermal lesions, which are easily sampled and studied, the detection of antibodies plays a smaller role than in BDV diagnosis. However, and especially in the asymptomatic reservoir hosts, OPV antibodies have been detected with the haemagglutination inhibition test, neutralization assay, IFA, and competitive ELISA (69, 111, 202, 317).

2.4.2 Viral kinetics and its detection

CPXV tends to cause a localized infection in humans, although viraemia can occasionally be detected (224). The lesions contain large titres of virus, especially in the vesicular stage, so the best material for the detection of CPXV — by electron microscope, virus isolation, immunostaining, and PCR — is a biopsy or swab of a lesion. The same applies to animals, although feline infections in particular are more commonly generalised than those of humans. Patients are often presented at a late, eschar stage of the disease, when the virus titre may be low but still detectable with PCR. Histopathological lesions, especially eosinophilic A-type inclusions, are characteristic in the early course of the disease and may be sufficient for a cowpox diagnosis in cats, but specific methods for orthopoxviruses are needed for a verified diagnosis at least in humans, who seldom present with the pathognomonic inclusions (172, 244, 325).

Reliable diagnosis of BD is impossible *intra vitam* (271). RT nested PCR methods have amplified the viral genome from peripheral blood, but the results are of low diagnostic value, as numerous asymptomatic animals and humans are also reported to harbour BDV RNA in blood (reviewed in (158)), and BDV viraemia overall is controversial (299). RNA in blood might, however, be indicative of Borna disease if pursued very carefully, avoiding contamination, and when combined with the clinical picture and antibody detection. In addition, the detection of BDV antigen in the peripheral blood has been reported, but has been controversially discussed (see "2.4.1 Immunity and detection of serological response"). The definitive BD diagnosis is possible only *post mortem* by demonstrating the virus or viral markers in the brain (271). Virus isolation is the most reliable confirmatory method, although very slow and complicated by neurotropism of the virus and by an often very low virus titre (76, 118, 186). The diagnosis can be made several weeks earlier if based on the detection of viral antigen with immunohistology, and/or viral RNA with *in situ* hybridization or RT PCR (186, 271). As usual with such noncytopathogenic, cell-associated, persistent virus infections (9), the results from different methods are not always congruent, reflecting

the low viral amount (186). BDV is most often present in the olfactory bulb, caudate nuclei, hippocampus, lateroventral cerebral cortex, and medulla oblongata (186), so these represent the most important sampling sites for BDV detection.

2.5 TREATMENT AND PREVENTION OF COWPOX AND BORNA DISEASE

Supportive and symptomatic treatments so far remain the only authorized treatment for both cowpox and Borna disease. For cowpox, sterile bandages and antimicrobial drugs may be necessary to control secondary bacterial infections, and minor surgical interventions have occasionally been performed in the late phase of the infection (23, 313); corticosteroids are contraindicated (18, 23, 243, 335). Anti-VACV gammaglobulin has been used for severe human cases (23, 93, 329).

CPXV among other OPV infections have been prevented or attenuated by smallpox vaccinations in humans in the past (101). As always, the protection is not full, at least not in all individuals, as CPXV infections have occurred despite recent vaccination (23). In fact, CPXV may evade the existing immunity by its direct deposition in skin (23). However, reports of CPXV infections have markedly risen following the cessation of smallpox vaccinations (330). Whether this is the result of a rise in the true incidence or due to increased attention remains to be determined. With another OPV infection, monkeypox, a 20-fold increase in incidence during 20 years has recently been demonstrated (263). Fortunately, CPXV seems to be of low infectivity to humans (23).

In addition to humans, a VACV vaccine has been authorized and used to protect exotic animals from cowpox in Germany (reviewed in (182)), and experimental feline vaccinations have been pursued (208).

Cidofovir, a nucleoside analog, is effective against CPXV and several other DNA viruses (12). As its use is complicated by rigorous side effects, other effective antivirals have been rationally searched for, driven largely by the threat of smallpox virus use as a biological weapon (7). The most promising candidate antiviral is the compound ST246, which interrupts the maturation of OPV particles into enveloped viruses. ST246 is effective against VARV, MPXV, ECTV and CMLV, in addition to CPXV (293). It has inhibited lethal challenge in rodent experiments (255), and has been successfully used in conjunction with cidofovir and intravenous vaccinia immunoglobulin for the treatment of a severe human case of eczema vaccinatum (329). Another promising antiviral is CMX001, a less toxic derivative of cidofovir (258).

As severe losses were suffered and even more were threatened, vaccination against BDV was applied. When inactivated vaccines were shown to be ineffective, virulent

virus was applied subcutaneously (92, 194). Rigorous vaccination was compulsory in the former German Democratic Republic in 1962–1992, but the campaign proved ineffective. Nowadays, infections caused by these unsuccessful vaccinations possibly contribute to the fact that the incidence of clinical Borna disease is still highest in horses in Germany (90, 194); the same is slightly indicated by BDV molecular epidemiology (179). No vaccination against this persistent infection has since been recommended (90, 194), although new recombinant vaccine candidates expressing BDV-N mediate protection from the disease, but not from infection in rats (136).

Amantadine sulphate is an antiviral affecting influenza viruses and also showing some CNS effect for example in Parkinson's disease. Its use in the treatment of Borna disease has been debated (43, 46, 70, 85-87, 129). As amantadine sulphate is rather safe and readily available, it is used at the dose of 2–4 mg/kg for a minimum of 12 weeks in the treatment of veterinary patients showing aggravating symptoms. This is despite the lack of controlled and blinded studies on its effects (85, 165). Intracerebrally administered ribavirin interferes in BDV replication and results in clinical improvement, although not in viral clearance in rodent, but its use is compromised by side effects (166, 187, 215, 296). Some other antivirals have also been developed and tested against BDV, but they have so far remained in the initial phase (15, 16, 247, 254, 323).

Because of the lack of effective antivirals and vaccines, the prevention of contact with possible virus sources remains the only way to affect the incidence of BDV. Exact guidelines cannot be provided, as transmission routes and reservoirs remain to be defined, but as the transmission mode seems to be at least oronasal, care should be taken to avoid getting secretions or excretions from possibly diseased or reservoir animals onto mucous membranes. In addition, improvements in general hygiene in animal premises and the separation of horses and sheep have provided some protection, but have not led to the elimination of BDV (194).

As for cowpox, although it is not very contagious, lesions contain high amounts of virus and should therefore be covered, or their handling with bare, especially abraded hands avoided. A simple piece of advice, which most probably protects against both CPXV and BDV infections, would be to wash the hands with soap after a contact with rodents or any animal secretions, excreta or lesions, before touching the nose, eyes or mouth. If this is difficult, gloves or a respirator may help to minimize the mucosal contact, and consequently viral transmission.

3. AIMS OF THE STUDY

CPXV and BDV were previously known or suspected to infect humans and several other species in Europe, but their existence in Finland was unknown and diagnostic methods absent. Furthermore, wild rodents had been known to act as CPXV reservoirs, but the frequency, species diversity and geographic distribution of the infection were insufficiently known. As for BDV, wild rodents had been suspected to function as a reservoir, but this hypothesis had been insufficiently tested. It had also been commonly hypothesised that OPV antibodies and their prevalence resulting from smallpox vaccination would be waning in humans as a consequence of the cessation of smallpox vaccination, and this could affect the severity of the disease course. This had not, however, been demonstrated. The general aim of this thesis was, using various approaches, to address these issues, i.e. the importance of CPXV and BDV in Finland and in possible host species. The specific aims were to:

- Establish relevant methods for the detection of CPXV and other OPVs as well as BDV in Finland (I-IV);
- Determine whether CPXV and BDV exist in Finland, and if so, in which species (I and II);
- Discover how common OPV immunity is in different age groups in Finland (I);
- Characterize possible disease cases and clarify their epidemiological context (I);
- Establish the hosts and possible reservoir species of these viruses and their geographical distribution in wild rodents (I-IV);
- Elucidate the kinetics of BDV infection in the bank vole (IV).

4. MATERIALS AND METHODS

Most materials and methods are described in the original publications I–IV, either on print or as a supplementary material available via the Internet. In the latter case, the link is provided in the printed publication and the material printed as an appendix. Some methods not explained in detail in the original articles are described here more thoroughly. Also, some additional methods are described.

4.1 SAMPLING AND SAMPLE STORAGE

4.1.1 Ethical permission

National regulations were followed in the trapping of small mammals (I–III). The Ethics Committee of the University of Helsinki approved the use of human and animal samples and virus isolation protocols in studies I, II and III, and the County Administrative Board of Southern Finland approved the facilities and the protocol for study IV. Veterinarians signed informed consent forms for the use of their blood samples in studies I and III.

4.1.2 Human samples

A total of 499 human serum samples were either received from the diagnostic laboratory of Helsinki University Hospital, involving patients suspected to have Puumala hantavirus infections (III), or specifically collected at the Annual Veterinary Congress (Eläinlääkäripäivät) 2001 (I, III). In addition, two whole blood samples were investigated (III). Furthermore, a biopsy sample from a skin lesion together with several serum samples were obtained from a hospitalised patient (I), and a DNA sample, previously extracted from a pock lesion on a young girl in 1989, was provided by the National Institute for Health and Welfare (I).

4.1.3 Samples from domestic animals

For Borna research, serum samples from neurologically or behaviourally symptomatic horses (n = 108), cats (n = 30), dogs (n = 89) and sheep (n = 2) from different parts of Finland were received from veterinarians (III). In addition, samples from asymptomatic horses (n = 500), cats (n = 293), dogs (n = 6), cattle (n = 52), sheep (n = 20) and a rabbit (n = 1) were collected for BDV and/or OPV screening, either as control material or as

specific targets near a patient case (I, III). Fresh tissue samples were available from 4 of these horses and from 1 cowpox-suspected cat, and cerebrospinal fluid (CSF) from 11 dogs; moreover, whole blood was analysed for BDV RNA from 48 horses, 59 cats and 27 dogs (III, unpublished). A parallel study investigated paraffin-embedded, archival brain samples of horses and cats for BDV antigens (141).

4.1.4 Samples from wild mammals and birds

Small wild mammals were collected by snap-trapping (I-III) or live-trapping followed by blood sampling and release (III), euthanasia by cervical dislocation (III), or housing them in a laboratory, where they gave birth to the animals in the infection experiment (IV). Small mammal samples consisted of sera and whole blood samples collected from retro-orbital sinus with capillary tubes (I, III, IV), and of blood samples collected from the pulmonary cavity *post mortem* in PBS (I-III; (250b, 289b, 319b)). The trapped small mammals were predominantly rodents, which in Finland mainly consisted of bank voles (I, IV), but also included many other rodent species (II, III) and some common shrews (*Sorex araneus*) (III). The carcasses were set on dry ice and stored at either at -20 °C or -80 °C until aseptically autopsied and sampled (I–III). The laboratory bank voles were necropsied and sampled immediately after euthanasia (IV). In total, 1600 Finnish, 437 Buryatian (from Baikal region, Siberia, Russian Republic of Buryatia) and 270 German wild small mammals were trapped and used for BDV or OPV studies, or both (I–III, and Kinnunen et al. unpublished).

Blood samples from 292 grouse birds (*Tetraonidae* spp.) were gathered by hunters by drying blood in filter paper and later dissolving it in Dulbecco's PBS (III; (181b)). Wild predator samples consisted of blood samples from 3 wolves (*Canis lupus*) and 145 lynxes (*Lynx lynx*; III), collected from legally hunted animals by the Finnish Game and Fisheries Research Institute and stored at -20 °C. In addition to BDV IgG screening (III), these predator samples were also tested for the presence of OPV antibodies (Kinnunen et al. unpublished).

4.2 CONTROL MATERIAL

As BDV-positive control sera, the following material was used: polyclonal rabbit anti-N and -P rabbit sera (164), the monoclonal anti-N "Bo18" serum (121) (kindly provided by Prof. Peter Stäheli, Freiburg, Germany), as well as sera from experimentally and naturally infected cats (164), an experimentally infected rat, and horses with natural, verified Borna disease (kindly provided by Dr. Sibylle Herzog, Giessen, Germany). Sera from uninfected rabbits, cats and horses were utilised as negative controls. Plasmids containing BDV-N and -P sequences for use in the production of recombinant antigens were gifts from Prof. Mikael Berg, Uppsala, Sweden (35). (III, IV)

Control material for BDV RNA and antigen detection included BDV/He80-infected and non-infected C6 cells (kindly provided by Prof. Mikael Berg, Uppsala, Sweden), BDV-infected and non-infected cat brain tissue (196), as well as paraffin blocks from verified Borna diseased horses and experimentally infected mice and rats (gifts from Dr Jürgen Hausmann, Freiburg, Germany, and Prof. Christiane Herden, Hannover, Germany). Pernilla Syrjä (Helsinki, Finland) and the Animal Experiment Department of the University Animal Hospital kindly provided the brain samples from uninfected horses, cats and rats.

For the establishment and checking of CPXV methods, serum from smallpox-vaccinated and non-vaccinated colleagues, CPXV-infected and uninfected rodents (kind gifts from Dr Malcolm Bennett and Dr Kim Blasdell, Liverpool, UK, and Dr Donata Kalthoff, Insel Riems, Germany) and of VACV-infected calf (kindly provided by Dr Irja Davidkin, Helsinki, Finland) were used, in addition to several VACV strains (also from Dr Davidkin; I, II) and a plasmid expressing the partial VACV HA gene as DNA controls (253) (II). After isolation of the first Finnish CPXV strain (I), it was used as the basis for serological studies (I, II). Tissue samples from specific pathogen-free laboratory mice were included as negative controls, as well as HEX-labelled enhanced green fluorescent protein gene added to the samples as an inhibition control ((151b); II).

4.3 VIRUS ISOLATION

All the virus isolation attempts were performed in biosafety level 3 laboratories of the University of Helsinki Meilahti campus following strict safety precautions.

4.3.1 Cell cultures

Orthopoxvirus was isolated from tissue samples using the following protocol: A biopsy sample from a human patient's pock lesion, or the lung or liver or both from PCR-positive rodents were homogenised with a mortar and pestle, suspended 1:10 or 1:20 in the cell culture medium, i.e. modified Eagle's Medium (MEM) supplemented with 2% foetal calf serum (FCS), 0.3 mg/ml glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin, and 0.25 µg/ml amphotericin B, and pipetted onto almost confluent Vero E6 cells without other media. The suspension was incubated for 1 h at 37 °C in an atmosphere containing 5% CO_2, after which the rest of the medium was added on two occasions, one day apart. The cells were further incubated in similar conditions and checked daily for cytopathic effects, the culture medium was changed once a week and the cells split 1:5 once per two weeks. Through splitting, samples of cells and inoculum were also stored at -70 °C for possible PCR, EM and further cultivation studies, and cells were spotted on IFA glass wells for immunofluorescence assay. (I, II)

BDV isolation from rodent samples was attempted as described above in Vero E6 cells, and in addition, in the following cell lines: rat astrocyte C6, mouse neuroblastoma Neuro-2A, human neuroblastoma SK-N-SH, and in primary rabbit embryonic brain (REB) cells. REB cells were prepared from newborn, guillotine-killed rabbits by first suspending the brain with a mortar and pestle in cell culture medium and then homogenising the suspension with a 20G needle and syringe before inserting into laminin-coated (1.2 μg/cm^2) cell culture flasks. The cells were grown in 10% FCS-containing Dulbecco's modified Eagle's medium for about one week into 40–90% confluence before infection with 10% brain suspension as described above, and split once or twice 1:2. If the cells grew well, the FCS concentration was reduced to 5%, and if not, they were assisted with addition of 2–5 μg/ml laminin, and supplemented with fresh REB cells. Within a few weeks, before or when the REB cells stopped dividing, VeroE6 cells were introduced into the flasks. They rapidly overwhelmed the REB cells. The cultivation was continued as described above for 3–4 months. (Kinnunen et al. unpublished)

4.3.2 Experimental animals

A 20 μl volume of 10% or 1% tissue sample homogenate from OPV-PCR-positive rodents was injected intracerebrally (i.c.) into newborn NMRI mice within 24h of their birth. The mice were monitored daily and euthanized by cervical dislocation under isoflurane anaesthesia on day 6 p.i. The brains were collected and stored at -70 °C, followed by later homogenisation with 5000 rpm on the MagNAlyser instrument (Roche) using ceramic beads as a disruptor. Nucleic acids were extracted from the brain homogenates with a Viral DNA Mini kit (Qiagen) and OPV PCR was performed (see below). (II)

4.4 ELECTRON MICROSCOPY

Samples from the cowpox patient and virus isolation were dispensed on Formvar-carbon coated grids and negatively stained for 10–20 s with potassium phosphotungstate (2%, pH 6.0). The grids were studied under a JEOL 1400 transmission electron microscope (Jeol, Japan).

4.5 SEROLOGY

4.5.1 Immunofluorescence assay and avidity measurement

CPXV and other, cross-reacting, OPV-specific Ig(G)-antibodies were detected with an immunofluorescence assay (IFA) based on acetone-fixed, CPXV/FIN/T2000-infected Vero cells mixed with uninfected cells 1:10 as a simultaneous control (I, II). Titres of ≥20 for serum and ≥10 for whole blood were used as the cut-off. Some of the first results were also verified with a haemagglutination inhibition test.

BDV-specific antibodies were similarly measured with IFA based on persistently BDV/He80-infected, acetone-fixed C6 cells and uninfected C6 cells 1:2 (III, IV). Serum samples with a mean titre of ≥15 and blood samples with a titre of ≥10 were classified as positive. CSF samples were studied as undiluted. Preparation of BDV–IF glasses was optimised by changing several parameters and trying different treatments: the frequency and ratio of cell splitting before preparing the IF glasses, IF glass materials and treatments, fixation and permeabilization agents and duration, antigen denaturation with 8-M urea, and the ratio of infected and uninfected cells. Cultivation for one week before pipetting of one part of the infected together with two parts of the uninfected cells onto Teflon-coated 10-well glasses followed by 20 min fixation and permeabilization with acetone produced the best results.

Avidity measurement was used for estimating the age and specificity of antibody responses (139, 283). The avidity index (AIX) was calculated as (IFA titre with 6-M urea wash)/ (IFA titre without urea wash) x 100% (37), and antibodies with AIX ≥ 25% were interpreted as highly avid (I-III).

4.5.2 Verification of BDV antibody findings: recombinant antigens and peptide arrays

The most important BDV-IFA results were verified with other, independent methods. Recombinant BDV N and P antigens were produced as glutathione S-transferase fusion proteins in *Escherichia coli* (35), and constructed and produced in the baculovirus expression system in *Spodoptera frugiperda* (*Sf*9) insect cells (290), also used by (347). Recombinant antigens served in immunoblotting and *Sf*9 cell-based IFA for antibody detection (the appendix of III). Furthermore, epitope mapping using a peptide array covering the coding regions of BDV N and P genes was carried out (37) (The appendix of III).

4.6 WORK WITH GENETIC MATERIAL

4.6.1 Controlling the contamination risk

Extremely strict care was taken in avoiding contamination of samples by one-way flow through three to five separate laboratories and two buildings (II-IV). Finnish samples were stored and handled, and master mixes pipetted at special laboratories of the Faculty of Veterinary Medicine. For sample handling, workers dressed with particular protective clothes. Only highly diluted positive controls (RNA from 0.005–50 BDV/He80-infected cells, 120 copies of partial VACV HA gene for CPXV) were used in a laboratory different from the samples (II-IV). DNA, cDNA or the first PCR round amplicon were transported in tightly closed tubes 5–8 km to the Haartman Institute, where sample processing continued in separate laboratories wearing distinct protective clothes (II-IV). In all laboratories, detergent disinfectants and ultraviolet light were employed before and after any sample, reagent or control handling; only filter tips were used; and gloves were frequently changed.

4.6.2 Sample homogenisation and nucleic acid preparation

Tissue samples were homogenised with a mortar and pestle (I-III), Tissue Lyser (Qiagen; II) or in a MagNAlyser instrument (Roche; IV). Nucleic acids were extracted either manually with the Tripure kit (Roche; I, III, IV), the AllPrep DNA/RNA Mini kit (Qiagen; II, IV), or the High Pure PCR Template Preparation kit (Qiagen; II); or employing automatons using the MagNApure total nucleic acid kit (Roche; II) or the NucleoSpin 96 Virus Core kit (Macherey–Nagel; II). Nucleic acids were used as fresh or after storage for a few days at +4 °C (DNA) or longer at -70 °C.

For BDV PCR (III), fresh blood samples with ethylenediaminetetra-acetic acid (EDTA) as the anticoagulant were stored with 10% volume of dimethyl sulfoxide (DMSO) at -70 °C and RNA was later extracted from leucocytes with the Qiagen RNA Blood kit. Samples older than two days were stored as such at -70 °C and RNA extracted from 100 µl of whole blood with the Tripure kit (Roche).

4.6.3 PCR

Parts of the genomic OPV DNA were amplified with several PCRs (Table 5). These targeted the haemagglutinin gene (artus Orthopox LC PCR kit, Qiagen, and (73, 253, 265)) coding for a membrane protein related to viral entry (74, 218); the thymidine kinase gene (270), the product of which is involved in the synthesis of deoxyribonucleotides to enhance DNA replication in cells and located within

the conserved part of the genome (74, 218); the A-type inclusion body gene (213) located within the conserved part of the genome and encoding with high capacity the inclusion protein (74, 218) (I, II); as well as the 14 kDa (fusion) protein gene (234, 274) (II), the product of which belongs to a putative entry complex (74, 218).

RNA from BDV samples was reverse transcribed and amplified with primers annealing to the nucleotides 210–227 and 585–606 (outer round), and to 229–247 and 462–483 (inner round) of the BDV/He80 N gene sequence (Table 5; III, IV). In addition, methods for amplifying the P gene of BDV were utilised (34, 341) (IV). BDV-specific DNA was amplified with the methods described above without the revere transcription step. For some highly interesting samples, degenerated primers were planned and used to specifically anneal to the conserved transcription start and termination sites of BDV strains (S2, T2; Table 5) (249), but they proved insensitive (Kinnunen et al. unpublished).

None of the PCR methods were used in quantification of the target gene, i.e. normalised to a host gene and compared with a standard curve, but the methods were employed on a qualitative basis.

Table 5. *PCR methods used for the detection of cowpox virus (CPXV) / orthopoxvirus (OPV) and Borna disease virus (BDV) nucleic acids.*

Target gene	Product size, bp[a]	RT[b] primers / template	Forward primer	Reverse primer	Probes[c]	Reference
OPV HA[d]	179	-	"*poxHA1.1*" GTGATGATGCAACTC TATCATG	"*poxHA1.2*" TGTAACTAGATCATCG TATGGAGA	"*Pox-FL*" (anchor) CTAAAAGAATAATG GAATTGGGCTCC-f; "*Pox-LC*" (sensor) LCRed640-ATACCAA GCACTCATAACAACAT AATCATTTATATTAT-p	(253)
OPV HA	1171	-	"*HAOUTF*" CCATTG GAAAAAACACAGTAC	"*HAOUTR*" CCAAATATATTCCCAT AGTC	-	(73)
CPXV HA	629-677	-	"*CPV1*" ATGACACGATT GCCAATACTTC	"*CPV2*" CTTACTGTAGT GTATGAGACAGC	-	(265)
OPV TK[e]	339	-	"*TK1*" AAAAGTACA GAATTAATTAG	"*TK2*" TTCAGATAATG GAATAAGAT	-	(270)
OPV ATIP[f]	CPXV:1672, VACV:1596, ECTV: 1219, CMPV: 881	-	"*ATI-up*" AATACAA GGAGGATCT	"*ATI-low*" CTTAACTTT TTCTTTCTC	-	(213)
OPV 14 kDa protein	110	-	*"Orthopox-2F"* CCGX[g]/ACCAGTCTGX/ AACATCAATC	*"Orthopox-2R"* ACAAATX/AGAAAAGT GTTGTAAACX/GCAA	*"Orthopox-2FAM"* FAM-CAGAGATATCATAGC CGCTCTTAGAGTTTC-BHQ1	(234)
OPV 14 kDa protein	146	-	*"GF"* GCCAGAGATAT CATAGCCGCTC	*"GR"* CAACGACTAAC TAATTTGGAAAAAAA GAT	*"14-kD POX"* TTTTC CAACCTAAATA GAACTTCATCGTT GCGTT	(274)

BDV N[h]	397	p40OF, p40OR, oligo-dT$_{18}$	"*p40OF*" ATYAGGCA GAACGCAGTG	"*p40OR*" GTAGTGTAG CAGTCTCACCATG	-	III
BDV N	256	Amplification product from primers p40OF and p40OR	"*p40IF*" GCATTGTTA GACCAGTCACG	"*p40IR*" GTAATGAG CAACARTGGCTGA	-	III
BDV P[i]	442	p23OF, p23OR, oligo-dT$_{18}$	"*p23OF*" TGACCCAAC CAGTAGACCA	"*p23OR*" GTCCCATTC ATCCGTTGTC	-	(34)
BDV P	355	Amplification product from primers p23OF and p23OR	"*p23IF*" TCAGACCCA GACCAGCGAA	"*p23IR*" AGCTGGGGA TAAATGCGCG	-	(34)
BDV P	707	S2b, T2b, oligo-dT$_{18}$	"*S2b*" RAAAACAAT GAACAAACCA	"*T2b*" GGTATGATGTC CCAYTCATC		Kinnunen et al. unpublished
BDV P	88	PrtF and PrtR	"*PrtF*" GAACCCCTC CATGATCTCAGAY	"*PrtR*" CTCYGTCAC TAGCTTCTTGATRAG	"*PrtP*" FAM-CAGC GAACCGGAAGG GAGCAGCTATC-BHQ	(341)

[a] bp, base pairs
[b] RT, reverse transcription
[c] Probes used for real-time PCR
[d] HA, haemagglutinin gene
[e] TK, thymidine kinase gene
[f] ATIP, A-type inclusion body gene
[g] X, inosine
[h] N, nucleoprotein gene
[i] P, phosphoprotein gene

4.6.4 Sequencing and sequence analysis

The amplicons were purified with the Qiagen Gel Extraction kit (I, II), the Qiagen PCR Purification kit (I and Kinnunen et al. unpublished) or the enzymes Exonuclease I and SAP (Fermentas; IV). The resulting DNA was cycle sequenced in both directions with Big Dye Terminator reagents (Applied Biosystems) in an ABI 3130xI capillary sequencer device (I–IV).

Forward and reverse sequences were checked against each other and verified by the chromatograms. Then they were analysed with the BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html) with a further search for matches using BlastN (http://blast.ncbi.nlm.nih.gov) (I, II, IV), and alignment using ClustalX (http://www.clustal.org), continued by maximum likelihood analysis with the software TREE-PUZZLE (http://www.tree-puzzle.de), the results of which were compared to those from the programs of the PHYLIP package (http://www.phylip.com) (I, Kinnunen et al. unpublished).

4.6.5 Verification of DNA findings

BDV DNA findings were verified by comparing the PCR results with and without previous template digestion with DNAse and RNAse (IV).

4.7 HISTOLOGY AND IMMUNOHISTOLOGY

Paraffin-embedded, formalin-fixed tissue samples were subjected to histological haematoxylin-eosin staining and immunohistological staining with a 1:5000 dilution of polyclonal rabbit anti-BDV-N or, as a control, preimmune antibody (164), mouse monoclonal anti-N antibody Bo18 1:100 (121) or a polyclonal rabbit anti-BDV-P antibody 1:20 000 (164). The staining was performed utilising the avidin-biotin-peroxidase system (185) either manually, including an antigen retrieval step with protease and an overnight incubation at +4°C ((141); Kinnunen et al. unpublished), or in a Ventana Discovery Automatic Slidestainer (Ventana Medical Systems; IV). Cleaved caspase-3 expression was demonstrated according to a previous protocol (161).

4.8 EXPERIMENTAL INFECTION OF BANK VOLES

Thirteen bank vole dams, which were originally born to wild-caught parents trapped by researchers at the University of Jyväskylä, and their 50 pups participated in the BDV infection experiment in the biosafety 3 level laboratory of the Faculty of Veterinary Medicine (IV). They were maintained under negative pressure in

individually ventilated, high-efficiency particulate air (HEPA) filtered cages (Isocage Unit, Tecniplast, Italy), the function of which was checked daily. The pups were infected i.c. with 10^2, 10^3 or 10^4 focus-forming units (ffu) of the "rat BDV" (303), or PBS as a control, and monitored daily until euthanasia at 2, 4, 6 or 8 weeks p.i. The cages were opened and animals handled in a biosafety changing station (Tecniplast, Italy) behind HEPA-filtered laminar flow.

Blood samples, and when possible, urine, were collected from live animals at the end of the study. Other samples, such as brain, organs, faecal-filled rectum (at least four times more faeces than tissue), and sometimes additional urine, were sampled from fresh carcasses, and stored for further use in immunohistology and PCR studies with the methods described above. (IV)

5. RESULTS AND DISCUSSION

5.1 ASSAYS ESTABLISHED FOR CPXV AND BDV DETECTION

Several methods for the detection of CPXV/OPV and BDV infections in various samples of animal and human origin were either set up according to the literature or designed and established. Of these, virus isolation as the ultimate proof of a viral infection was first set up. A functioning cell culture method for CPXV isolation with electron microscopic confirmation was established (I, Fig. 8), although it was insensitive to vole samples (II). Furthermore, numerous methods, such as neuronal cell lines and primary REB cells, were set up and used for BDV isolation attempts (Kinnunen et al. unpublished).

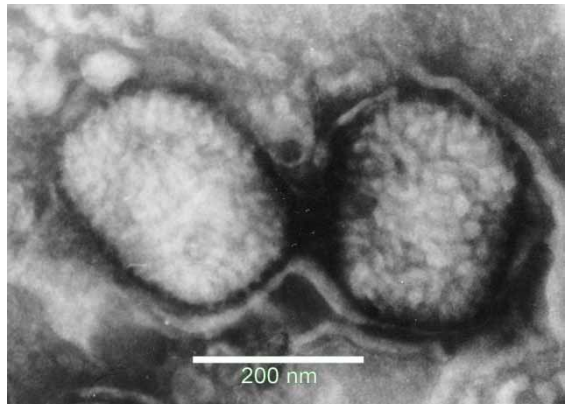

Figure 8. *Electron micrograph of the CPXV isolate FIN/T2000 originating from a cowpox patient. (Original figure: Inki Luoto, HUSLAB, Helsinki; with permission.)*

Secondly, immunofluorescence assays (IFA) based on the newly isolated Finnish CPXV isolate T2000 and the BDV strain He80, as well as BDV recombinant antigens N and P, were established for OPV / BDV antibody detection from blood, serum and CSF samples of humans and animals (I, III). The IFA method was enriched to include the measurement of antibody avidity in order to differentiate acute from past infections, and also to assess the specificity of BDV antibody findings (I, III). For further verification of BDV antibody results, immunoblotting and peptide arrays were set up (III).

Thirdly, several conventional and real-time PCRs including sequence analysis (but not quantification) were established to detect CPXV and BDV genes in human and animal tissues and excreta (Table 5) (I–IV). The primers for the nested BDV PCR targeting the N gene were planned to anneal to all available BDV sequences at the time of establishment, including the most variable No98 subtype (228). The repeatable sensitivity of that newly established method was 1/160 of a persistently BDV-infected cell and 1 spiked infected cell in 90 µl of feline blood (Fig. 9). Although sensitive for BDV control strains, the primers are not necessarily optimal for other deviant bornaviral sequences; for example, they do not detect the recently found ABVs (176) *in silico*. A typically low BDV amount in infected tissues comprises a further challenge. In this study, this biological fact was controlled by detecting the two most abundantly transcribed genes and the corresponding mRNAs, N and P, with high sensitivity (34, 341) (Fig. 9; III). Taken together, the BDV PCR methods employed were the best available when the study was conducted. In addition, exceptional care was taken to prevent possible contamination (49).

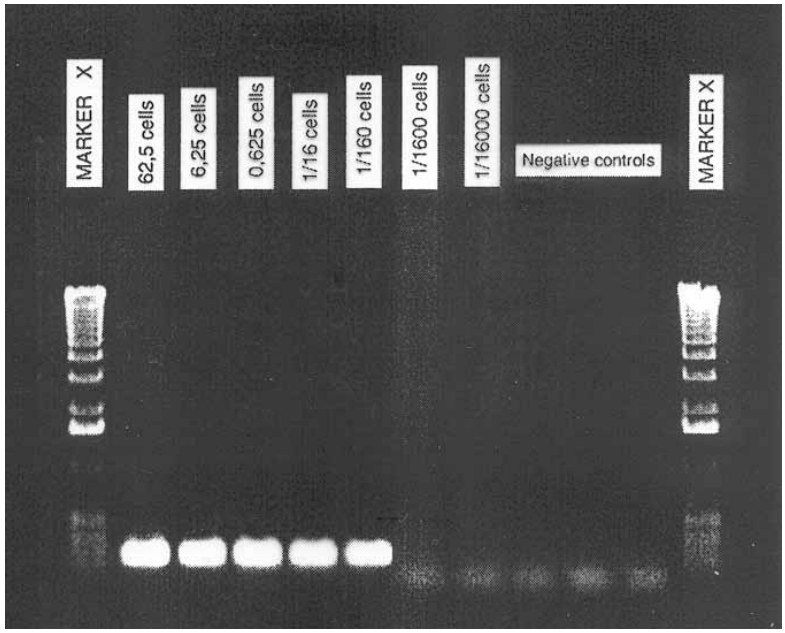

Figure 9. *The BDV RT nested PCR is sensitive: 1/160 of a BDV-infected cell is enough to detect N gene RNA.*

As the fourth type of detection method, immunohistology was established to evaluate the viral distribution and for the detection of a Finnish BDV variant possibly escaping PCR methods (IV). It is the most common technology used for *post mortem* confirmation of BDV infection in animals (271), and may also detect slightly

divergent virus variants without any risk of contamination. In some animals with overt neurological disease, however, only very few antigen-positive cells can be visualized, which indicates that BD can sometimes escape detection by immunohistology (57).

The most relevant methods for CPXV and BDV detection and diagnosis were established (I-IV). In addition to assays recognizing viral nucleic acids, viruses, and viral antigen (BDV), methods detecting antibodies and the avidity of antibodies became available. The employment of several independent methods is especially important for BDV infections, the detection of which has been hampered by contradictory results (194, 271). This is why special care was taken concerning specificity issues, probably at the cost of sensitivity. For instance, although IFA is considered the most reliable method for BDV antibody detection (reviewed by (299)), it may be 10x less sensitive than ELISA, and their BDV results do not always correlate in naturally infected cats (164). Many factors were noted to affect the IFA results: in addition to unknown factors, the fixation and permeabilization conditions, as well as the time from the splitting of cells played a role. BDV prevalences based on the IFA are most probably underestimated, as not all positive control sera from Germany and Sweden were positive, but all negative controls were negative. Some of the control sera from verified Borna diseased horses in Germany repeatedly gave positive IFA results with a 1:10 dilution only, so the cut off of 1:15 for serum was chosen. Previously reported cut-offs for IFA have varied from 1:5 to 1:20 (271, 321). Unfortunately, no serum panel originating from individuals detected as BDV-positive or -negative with verified, agreed methods, exist for exact validation of the sensitivity and specificity of any assay (271). Hence, to classify a sample as verified positive, we employed additional methods (avidity measurement, recombinant antigens, and peptide array) or repeated sampling, or both. The ultimate aim was to determine whether verifiable infections with BDV or a closely related agent exist in the target species and regions instead of studying seroprevalence with optimised sensitivity. For this purpose, the IFA, combined with the other serological methods, worked well.

The sensitivity of OPV IFA proved good when compared to the results from another IFA utilising CPXV-infected and uninfected rodent sera from Great Britain (Kinnunen et al. Unpublished), as well as vaccinated and unvaccinated human sera (I, Fig. 10). For cow samples, however, the method was not ideal, as a high proportion (but not all) of cattle sera showed unspecific reactivity to Vero cells. These samples were discarded from the analysis. A strict cut-off was applied for both IFAs: as compared to those classified as positives, at least the same (OPV) or double (BDV) the number of unclear results was seen and interpreted as negative.

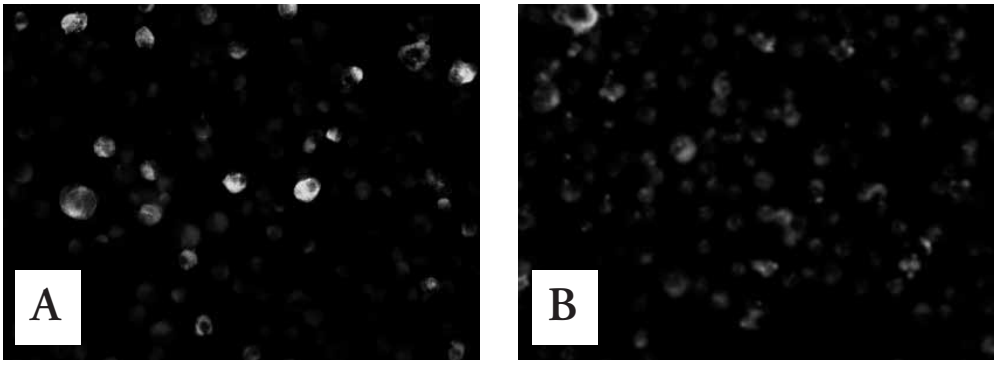

Figure 10. *An orthopoxvirus (OPV) IFA based on cowpox virus strain T2000-infected and uninfected (~1:10) Vero cells detects OPV-IgG antibodies in the serum of a vaccinia virus-vaccinated (A) but not of an unvaccinated person (B). Original magnifications 400x.*

Avidity measurement, in addition to IgM detection, is a valuable tool in the timing of primary infections in humans (139, 140). However, it could also be studied and utilised more on animals, as the diagnosis could be made earlier instead of waiting for the paired serum sample. To validate the method properly for each pathogen and species, serial serum samples should be studied from experimentally infected animals, similarly to a study on the response of bank voles to hantavirus (112), or from a well-known outbreak (19). In the OPV avidity assay, the results were as anticipated: those vaccinated or infected months or years ago had highly avid antibodies, whereas in acutely ill or clearly PCR-positive individuals or both, low avidity (or absence of antibodies) was seen (I, II). Since the findings of the BDV avidity assay could not be compared to the findings of methods detecting nucleic acid, antigen or virus (see "5.5 A few mammal species harbour BDV antibodies in Finland"), they remain preliminary, although individuals with both kinds of antibodies, either with high or low avidity, were reliably detected (see later; III). Nevertheless, a high avidity of BDV antibodies can be interpreted as additional proof of a specific reaction (8, 37), and was thus used as a verification method for the presence of BDV infections in Finland.

5.2 CPXV CAN CAUSE SEVERE INFECTION IN UNVACCINATED ATOPIC CHILDREN

Clinical samples from a few suspected cowpox patients were investigated. CPXV infections could be verified from two human patients by virus isolation, PCRs and sequencing (I). In a four-year-old, unvaccinated atopic girl from eastern Finland, cowpox (isolate T2000) manifested as severe, smallpox-resembling, generalised dermatitis necessitating one week of hospitalisation but resulting in a favourable outcome after some weeks (Fig. 11; I). This was the first verified cowpox case in Finland and proved the existence of CPXV in Finnish fauna. After that, a dermatitic,

17-year-old female patient from southern Finland was diagnosed from an archive lesion sample, dating back to 1989, to have suffered from cowpox (I, Kinnunen et al. Unpublished). This CPXV isolate was designated E1989. Both patients contracted cowpox in autumn, when the majority of cases have also been diagnosed in Great Britain and Germany (23, 172).

Figure 11. *Cowpox lesions on a patient's back and upper arm seven days after the onset of symptoms. Photo: Kyllikki Tarvainen, with permission.*

The female gender and young age of both the patients were unsurprising: 26% of cowpox cases have occurred in children <12 years old and 20% in young girls (22). Although cowpox usually manifests as one or two skin lesions also in children (23), people specifically suffering from atopic dermatitis or allergic rhinitis have had severe generalised cowpox eruptions resembling that of the Finnish girl ((40, 154); reviewed by (23)). Similarly to the Finnish case, some difficulties have arisen before making the correct diagnosis, and the lesions in these patients have likewise recovered uneventfully in around four weeks after the onset of the symptoms. The infection source has remained unresolved in 46% of the cases (23), but most often contact with a sick rat (154) or cat (40) has been suspected or proven (see the literature review). The girl from southern Finland owned a cat, which was antibody-negative when the patient's disease was acute (Kinnunen et al. unpublished). In the case from eastern Finland, the girl had played with a dead bat, which was not available for the studies, but two dogs of the family showed an OPV antibody response (I; Table 6). As expected, the dogs were healthy at the time of clinical examination, several months after the disease in the girl. OPV infection in dogs seems to be rare, as few previous and only one recent report are available (291, 326, 327). Overall, OPVs are not apparently very pathogenic

to carnivores, as evidenced by the mildness of the natural canine and experimental fox infections (50). Thus, a dog had most probably transmitted the virus from an unnoticed lesion, although the role of the bat or direct contact to rodents or their excreta could not be excluded. No published evidence exists on CPXV infection in bats.

5.3 OPV ANTIBODIES ARE RARE IN YOUNGER AGE GROUPS

To assess the OPV antibody prevalence in different age groups in Finland, 138 veterinarians were sampled during the Annual Veterinary Conference (Eläinlääkäripäivät) 2001. A lower OPV antibody prevalence in younger age groups was seen in the panel (I and Fig. 12). This corresponds well with the gradual cessation of smallpox vaccination with VACV, which is cross-reactive and -protective against CPXV in addition to smallpox virus. In those born in 1972 or later, the prevalence and thus protection could be seen in less than 18% of the individuals, indicating an increasing susceptibility to cowpox and any OPV infection. (I)

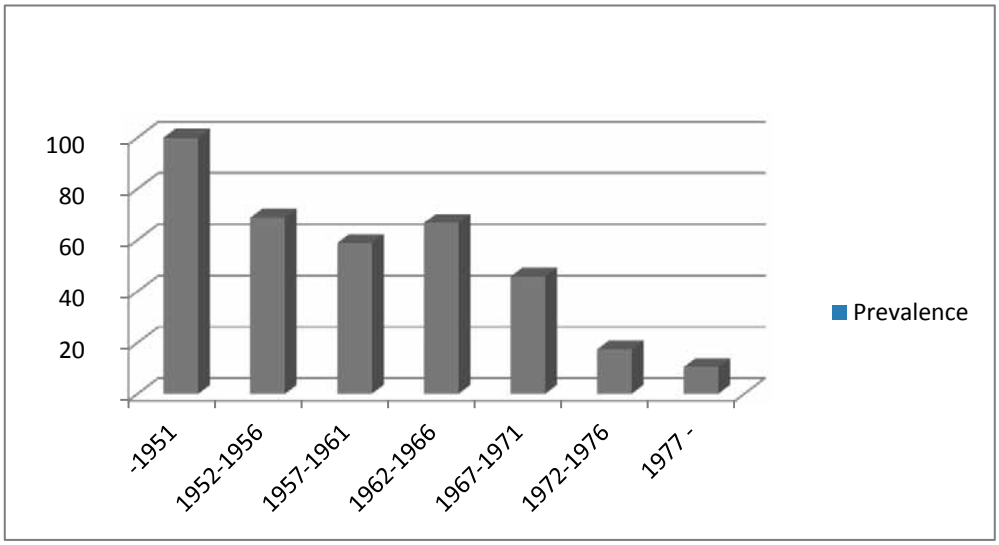

Figure 12. *Orthopoxvirus antibody prevalence (%) in veterinarians in 2001 according to the year of birth, as determined by immunofluorescence assays.*

In addition to reflecting the cessation of VACV vaccinations, these results indicate that at least two seropositive individuals in the youngest age group had most probably responded to natural CPXV infection, as the last VACV vaccinations were carried out in 1977 when even the oldest individuals in that group were too young to be vaccinated: the recommended age for the first vaccination has been one to two years (79). These findings were rechecked with veterinary samples collected in 2009 showing that the overall prevalence had decreased as expected, because later age groups had entered the veterinary profession (Kinnunen et al. unpublished). Again, some antibody responses seemed to arise from natural infection instead of vaccination. The average population without frequent animal contact may even have a lower OPV antibody prevalence and is therefore vulnerable to cowpox, and in the improbable case of a deliberate release, to smallpox. This emphasises the importance of public awareness, at least among people with atopic diseases who are therefore prone to severe manifestations, and among veterinarians, who frequently encounter sick cats or their samples. Fortunately, cowpox was nominated as an immediately notifiable animal disease and immediately notifiable zoonosis in the veterinary legislation in 2010. Veterinarians are, indeed, the key players in inhibiting zoonotic transmission from domestic animals to humans, including themselves.

Orthopoxvirus protection usually lasts long after vaccination: more than 90% of people vaccinated 25–75 years ago still maintain substantial immunity against VACV ((130); reviewed in (74)). The antiviral antibody response remained stable 1–75 years post-vaccination, whereas antiviral T-cell responses declined with a half-life of 8–15 years. Whether the cessation of smallpox vaccination campaigns will lead to an increased OPV disease incidence has long been discussed, and some researchers hypothesise that VACV vaccinations provide only limited and uncertain protection against cowpox (22). However, the increasing OPV disease incidence assumed elsewhere (330) has now been proven: the cumulative incidence of monkeypox increased 20-fold from the 1980s to the years 2006–7 in the Democratic Republic of Congo, where unvaccinated persons have a 5.2-fold higher risk of monkeypox than vaccinated persons. The incubation time and severity of cowpox symptoms have also been associated with the patient's VACV vaccination status in the family: two unvaccinated girls had multiple lesions accompanied by local lymphadenopathy and fever after only 3 and 5 days of incubation (55). In contrast, the incubation period for three VACV-vaccinated adults was longer than 7 days, and they presented with a single small lesion without any other symptoms. A similar, milder clinical picture has been described in a vaccinated man (68). These cases indicate that VACV vaccination conveys partial protection against CPXV, but they are too few to draw final conclusions.

5.4 WIDE MAMMAL SPECTRUM HARBOURS OPV ANTIBODIES IN FINLAND

The presence of OPV infections was examined with IFA screening of hundreds of blood samples from vertebrate animals resident in Finland. In the cat, dog, horse, cow and lynx, antibodies to orthopoxviruses (including CPXV) were detected (I, Kinnunen et al. unpublished; Table 6). Most of the OPV antibody-positive animals lived in southern Finland, and the antibody avidity was high in the majority of the samples, indicating infection weeks to years ago (data not shown).

The large host spectrum is different from that of other poxviruses but typical of CPXV (98). However, it was noteworthy that horses commonly had OPV antibodies (3.1%). The most probable explanation would be subclinical infection after exposure to wild rodents or their excreta. Nevertheless, it cannot be excluded that infected horses become sick. Are some of the mysterious skin lesions occasionally encountered in horses caused by CPXV? A single clinical cowpox case in a horse has been verified in Germany this century (243). The patient suffered from papular dermatitis all over the body, crater-like tongue and mucosal lesions, purulent conjunctivitis, anorexia and apathy. Full recovery was, however, rapid, taking only a few days. Remarkably, no other horse or other animal in the circus where the horse came from became sick, and as this is the only report of cowpox in horses, it should be regarded as an exception probably based on individual predisposition. In the 19th century, however, so-called horse-pox was common in horses but later disappeared (197). It was a generalised, three-week-long dermatitis, which was contagious to other animals and man, in the latter resembling smallpox vaccination lesions.

The finding of OPV infections in lynxes verified previous results obtained from a more sensitive but possibly not so specific method, competitive ELISA (314). In the previous study, 1 of 73 Finnish and 5 of 17 Swedish lynxes harboured OPV antibodies, so the prevalence in Finland has remained the same (currently 1.4%). The seropositive lynxes were hunted in eastern Finland, one of them even in the very municipality in which the human cowpox case was detected. Wild carnivores seem to be susceptible to the infection and respond to it by producing antibodies, as also evidenced by experimental (50, 51) and natural infection of foxes (208, 314) and a brown bear (314).

In addition to western and eastern Finland, OPV infections were common in cats living in the capital region (up to 13.7%). In Great Britain, 32% of the cats with verified cowpox virus infection also lived in suburban or urban environments (31). For a small proportion (8/16) of the seropositive individuals from the capital region, the descriptions (signalement) were available. Six of them were males and two females, the median age was 8.5 years and all but one represented mixed breeds. Seven cats lived in Helsinki and one in nearby Espoo; at least four of them were from locations where cats are commonly left to roam free in a semi-urban environment. The rest had

probably acquired the infection either at other habitats or during a stay at a summer cottage. Five of the cats had become infected in the past, as their antibodies had high avidity, whereas three cats presented with low-avid antibodies indicating a more acute infection. The probably acutely infected cats had been sampled in May, October, and November, all months with a known cowpox incidence (172). No clinical data were available from these cats, but these findings emphasise the importance of also remembering cowpox as a differential diagnosis in cities, and consequently sampling skin and mucosal lesions for OPV detection. As cats are the most common infection source for humans (23), clinical suspicion and a specific diagnosis are important to prevent zoonotic transmission. During this study, only one clinical suspicion was apparent, but the cat was PCR- and antibody-negative, and later proved to have T-cell lymphoma (Kinnunen et al. unpublished). Previous cat studies from other countries have revealed OPV antibody prevalences of 0–2% in Great Britain (31, 111, 345), 0.46–10% (lower prevalence with IFA, higher with competitive ELISA) in Norway (316), 0.41–13% in Germany (208, 225, 349), and 2% in Austria (225). In addition to the geographical distribution of feline CPXV/OPV infections, prevalence differences may be explained by the different methods used: competitive ELISA seems to be considerably more sensitive than IFA (316), and haemagglutination inhibition is slightly more sensitive than the virus neutralization test (29, 111). No previous data are available on the development of OPV antibody avidity or the longevity of IFA antibodies in cats. If, as is probably the case, they reflect those from humans (I; (130)), and those detected with other methods in cats (from 6–16 days post infection (dpi) onwards, for a minimum of 6 months; (29, 32, 111)), the low-avid antibodies would indeed be a sign of an acute infection and the antibodies would remain for months to years, even decades, after infection. Thus, the high OPV seroprevalence in cats (Table 6) would reflect the proportion of cats that had encountered a CPXV infection 6 days to years previously.

Interestingly, one cow was seroreactive to OPVs (Table 6). The cow originated from a large farm in southern Finland, some cows from which presented acute farmyard pox lesions (parapoxvirus infection) at the time of sampling. Since the antibodies did not correlate with parapoxvirus findings, they are indicative of the exposure of Finnish cattle to OPVs in addition to the better known farmyard pox. Further studies are needed to assess the clinical relevance of this preliminary finding, but as the last reported clinical cowpox cases in cattle date back to the 1970s and happened at a farm with poor milking hygiene and udder health (114), the relevance most likely remains exiguous.

Table 6. *Orthopoxvirus antibodies in large Finnish mammals as detected by immunofluorescence assays using cowpox virus as the antigen.*

Panel	n	OPV positive	Prevalence, %	CIs for prevalence[a]	Publication
Cat, total	**213**	**21**	**9.9**	***6.21 – 14.7***	
From the same farm as a cowpox patient	1	0	0		Unpublished
Southern Finland	117	16	13.7	*1.91 – 10.8*	Unpublished
Stray cats in the capital region	77	3	3.9	*0.811 – 11.0*	I
Western Finland	6	1	17	*0.421 – 64.1*	Unpublished
Eastern Finland	12	1	8.3	*0.211 – 38.5*	Unpublished
Dog, from same farm as a cowpox patient	3	2	67	*9.43 – 99.2*	I
Horse, total	**223**	**7**	**3.1**	***1.27 – 6.36***	
From same farm as a cowpox patient	1	0	0		I
Southern Finland	111	5	4.5	*1.48 – 10.2*	I, Unpublished
Western Finland	8	0	< 13	*0.0 – 36.9*	Unpublished
Åland Islands	12	0	< 8.3	*0.0 – 26.5*	I
Northern Finland	12	0	< 8.3	*0.0 – 26.5*	Unpublished
Eastern Finland	79	2	2.5	*0.308 – 8.85*	I
Rabbit, from the same farm as a cowpox patient	**1**	**0**	**0**		I
Cattle	**12**	**1**	**8.3**	***0.211 – 38.5***	Unpublished
Lynx	**145**	**2**	**1.4**	***0.167 – 4.89***	Unpublished
Wolf	**3**	**0**	**<33**	***0.0 – 70.8***	Unpublished

[a] 95% confidence intervals, Fisher's exact test

5.5 A FEW MAMMAL SPECIES HARBOUR BDV ANTIBODIES IN FINLAND

IFA screening of more 1500 blood samples was used to investigate the presence of BDV infections in vertebrate animals resident in Finland. BDV-specific antibodies existed in cat, dog and horse blood samples, the highest prevalences being in stable mates of other seropositive horses and in recently imported horses, which were sampled for statutory equine infectious anaemia screening, as well as in neurobehavioural cat and horse patients (III, Kinnunen et al. unpublished; Table 7). Most seropositive horses and one of the two cats had high-avid antibodies, and furthermore, most IFA findings were verified with either a peptide array or recombinant antigens, or both (III). Although BDV antibodies were more commonly found in neurobehaviourally symptomatic horses and cats than in other animals, no significant correlation with the symptom status could be seen. Instead, stable mates of BDV-antibody positive horses had a significantly greater risk of being seropositive as compared to horses without such contact ($p = 0.0097$, Mid-P exact test), indicating exogenous infection from either each other or a common source rather than some kind of activation of cross-reactive antibodies. Interestingly, one symptomatic, BDV-antibody-positive horse had previously eaten oats from the very field where a BDV-antibody-positive root/tundra vole (*Microtus oeconomus*) was trapped suggesting but not proving a possible transmission chain (III). Previously, a prevalence of 33% has been found in horses from stables with a history of Borna disease (148, 260).

Like horses, a close proximity to BDV-antibody positive horses was a risk factor for a dog to also be BDV-antibody-positive ($p = 0.01630$, MidP exact test). The only dog presenting with antibodies was a clinically healthy hunting dog, whereas none of the neurobehaviourally symptomatic dogs had antibodies. Although BD can affect dogs, the infections and disease appear rare (232, 338).

In contrast to reports from Sweden, BDV infections were not found among Finnish birds or lynxes (36, 84). Furthermore, BDV infections did not exist or existed with low prevalence in sheep and cattle. Overall, the BDV antibody prevalences from serum were the same as or lower than elsewhere based on IFA: 2.7% in American and 29–100% in German horses (169, 260, 320); 16% in German sheep (320); and 7–34% in German and 17–44% in symptomatic Swedish cats (157, 196); reviewed in (158, 167). In these previous studies, species panels mainly much smaller (5–295 animals, median 27.5) than in the current study have been analysed, thus giving more influence to chance. In addition to IFA, BDV antibodies have also been detected with other serological methods, but as the results are quite incomparable with each other (110, 164, 168, 271), comparison of prevalences makes no sense.

Notably, BDV antibodies can only be found in the sera of 30–40% of verified, infected animals (119, 164, 171, 321), so these seroprevalences are clearly an underestimate

of the existing BDV infections. One explanation for the common lack of detectable antibodies could be the complexing of the antibodies with the corresponding circulating antigen (47), but as the existence of antigenaemia has been questioned (343), the special biology of BDV – the tight association with cells, neurotropism, persistence, and noncytopathogenicity – remains the most probable explanation for this kind of immune evasion (303).

Table 7. *Borna disease virus (BDV) antibodies in large Finnish vertebrates as detected by immunofluorescence assays using BDV strain He80 as the antigen.*

Panel	n	BDV positive	Prevalence, %	CIs for prevalence[a]	Publication
Cat, total	**323**	**2**	**0.62**	***0.075 – 2.22***	
Stray cats in capital region	74	0	< 1.4	*0.0 – 4.86*	III
Southern Finland	126	0	< 0.8	*0.0 – 2.89*	III, Unpublished
Western Finland	3	0	< 33	*0.0 – 70.8*	Unpublished
Eastern Finland	12	0	< 8.3	*0.0 – 26.5*	III
Healthy cats, all Finland	78	1	1.3	*0.032 – 6.94*	III
Cats without neurobehavioral symptoms, total	293	1	0.34	*0.008 – 1.89*	
Neurological / behavioural patients, all Finland	30	1	3.3	*0.084 – 17.2*	III
Dog, total	**92**	**1**	**1.1**	***0.027 – 5.91***	
From the same farm as a BDV-positive horse	3	1	33	*0.84 – 90.6*	III
Neurological symptoms	89	0	< 1.1	*0.0 – 4.06*	III
Horse, total	**608**	**12**	**1.97**	***1.02 – 3.42***	
Southern Finland	83	0	< 1.2	*0.0 – 4.35*	Unpublished
Western Finland	20	0	< 5.0	*0.0 – 16.8*	Unpublished
Åland Islands	12	0	< 8.3	*0.0 – 26.5*	III
Northern Finland	18	0	< 5.6	*0.0 – 18.5*	Unpublished
Eastern Finland	85	2	2.4	*0.286 – 8.24*	III, Unpublished
Recently imported	93	3	3.2	*0.671 – 9.14*	III, Unpublished
Virus infection suspects	53	0	< 1.9	*0.0 – 6.72*	III, Unpublished

Non-neurological patients, southern Finland	122	1	0.82	*0.020 – 4.48*	III, Unpublished
Horses without neurological symptoms or contact with BDV patient, total	486	6	1.23	*0.454 – 2.67*	
Neurological / behavioural patients	108	4	3.70	*1.02 – 9.21*	III, Unpublished
From the same farm as a BDV-positive horse	14	2	14	*1.78 – 42.8*	III
Cattle	**52**	**0**	**< 1.9**	***0.0 – 6.85***	III
Sheep	**22**	**0**	**< 4.6**	***0.0 – 15.4***	III
Lynx	**145**	**0**	**< 0.69**	***0.0 – 2.51***	III
Wolf	**3**	**0**	**< 33**	***0.0 – 70.8***	III
Bird, grouse	**292**	**0**	**< 0.34**	***0.0 – 1.26***	III

[a] 95% confidence intervals, Fisher's exact test

PCRs did not amplify specific nucleic acids from the skin biopsy of the single cowpox-suspected cat (Kinnunen et al. unpublished), or from any of the 134 whole blood or 4 brain samples of the BDV-studied horses or cats (III). It is exceptional to obtain proper tissue samples from clinicians for virus detection: brain for BDV and lesion biopsies or organs for CPXV. The lack of samples most probably explains why no PCR-positive patients were found in this study. On the other hand, a few BDV-antigen-positive animal brains have been detected among archived samples (Fig. 13) ((141) and Kinnunen et al. unpublished). Moreover, a transient BDV infection of REB cells inoculated with a cerebrocortical sample from an equine patient could be seen (240), although infectious virus could not be demonstrated in the (few) other animal samples. Difficulties in the isolation of BDV despite common antibody findings may be related to the markedly low level of virus production conveyed by the genome trimming strategy affecting viral but not RNA and protein amounts (279). BDV isolation has also previously proven demanding because BDV multiplies very slowly, is strictly neurotropic and, in the infected tissue, often has a very low titre (76, 118, 186) (and the review by (299)). However, BDV infections of many host species were proven by serological methods in this study, and two parallel studies also detected antigen (Fig. 13) and/or virus.

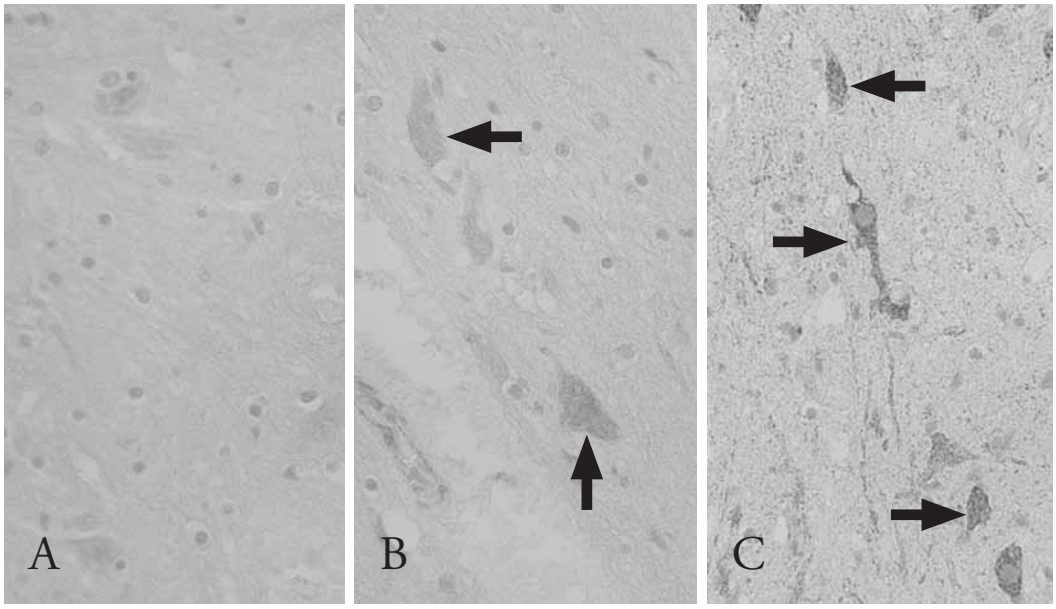

Figure 13. *An atactic cat with nonpurulent encephalitis expressing Borna disease virus (BDV) but not Puumala virus antigen in the cerebrum (arrowheads). Immunohistological staining with polyclonal rabbit antisera against glutathione S-transferase (GST) fusion proteins of (A) Puumala virus nucleocapsid protein (negative control) 1:5000, (B) BDV nucleocapsid protein 1:5000 and (C) BDV phosphoprotein 1:10 000. Magnification 400 x.*

5.6 OPV ANTIBODIES ARE COMMON AND BDV ANTIBODIES OCCASIONALLY FOUND IN EURASIAN WILD RODENTS

To find out hosts and possible reservoir species of CPXV/OPV and BDV infections and to elucidate their geographical distribution, more than 2000 wild small mammals from Finland, Germany and Buryatia, Russian Siberia, were sampled and studied for viral antibodies with IFA. Wild rodents had commonly had contact with OPVs in Eurasia: antibody-positive individuals were found in all three regions and in almost all parts of them (I, II, Table 8). The prevalences varied locally from 0% to as high as 92%, which was seen in one population in southern Finland (I). The few studied insectivores from Finland and Germany did not have OPV-specific antibodies (data not shown, Kinnunen et al. unpublished). Most of the seropositive rodents were bank voles, a well-known host for CPXV. However, especially in regions other than Finland, other known hosts, the field vole (*Microtus agrestis*), root/tundra vole (*Microtus oeconomus*), and gray red-backed vole (*Myodes rufocanus*) also harboured antibodies. In addition, three new CPXV/OPV host species were detected: the striped field mouse (*Apodemus agrarius*), reed vole (*Microtus fortis*) and striped dwarf hamster (*Cricetulus barabensis*) (I, II).

Table 8. *Orthopoxvirus antibodies in Eurasian wild rodents as detected by immunofluorescence assays using cowpox virus as the antigen.*

Panel	n	OPV-positive	Prevalence, %	CIs for prevalence[a]	Publication
Finland, total	**1445**	**142**	**9.83**	***8.34 – 11.5***	
South	295	82	27.8	*22.8 – 33.3*	I, II
West	649	52	8.01	*6.04 – 10.4*	I, II, Unpublished
North	465	7	1.51	*0.608 – 3.08*	I, Unpublished
East	36	1	2.78	*0.070 – 14.5*	I
Germany, total	**224**	**71**	**31.7**	***25.7 – 38.2***	
Nordrhein-Westfalen	27	0	< 3.7	*0.0 – 12.8*	II
Mecklenburg-Vorpommern	47	15	31.9	*19.1 – 47.1*	II
Sachsen-Anhalt	150	56	37.3	*29.6 – 45.6*	II
Buryatia, total	**437**	**14**	**3.20**	***1.76 – 5.32***	
Central	168	7	4.17	*1.69 – 8.40*	II
South	269	7	2.60	*1.05 – 5.29*	II

[a] 95% confidence intervals, Fisher's exact test

In these samples, OPV was more prevalent in Germany than in Finland or Buryatia (Table 8; II, Unpublished). As the rodents from different locations were not trapped at the same time, and the sampling was not planned to evenly represent whole countries, the figures are not completely comparable. These regional prevalence differences, however, are clearly statistically significant ($p < 0.00001$) further clarifying the fact that although generally present, CPXV displays considerable regional (96) and seasonal (63, 138) fluctuation in prevalence based on population demographical factors (25, 26).

The OPV species responsible for the antibody findings cannot be defined on a serological basis alone due to their cross-reaction. OPVs other than CPXV are not known in Eurasian wild rodents, although they occasionally infect rodents of Africa, North and South America (2, 74, 102). In laboratory mice, however, ECTV also exists in Europe, but evidence of natural infections is lacking (99). Furthermore, no species are known to be the principal host for more than one OPV (74). Thus, antibody findings in at least bank voles, which are naturally and experimentally susceptible to CPXV (30), are most probably caused by CPXV, as also are possibly antibodies in other rodents living in close proximity to bank voles. CPXV, indeed, seems to be

extremely ubiquitous in its host spectrum. In addition, its presence in other rodent species, both pet and wild rats, has resulted in transmission of the virus to zoo animals and humans (24, 154, 182, 205, 344). This study demonstrated that hamsters may also be hazardous in this respect, as is probably any pet rodent species.

For the first time, BDV antibodies were detected in wild rodents, namely voles in Finland (Table 9). Small numbers of wild rodents have also before and during this thesis project been tested without BDV findings (124, 150, 252, 319, 321). Of the seropositive individuals detected in this study, three were bank voles and one was a root/tundra vole (III and Kinnunen et al. unpublished). BDV could not be detected by RT nested PCR or virus isolation in cell cultures from the two tested voles (III and Kinnunen et al. unpublished). However, the existence of BDV-specific antibodies in rodents was verified by reaction with recombinant BDV antigens and BDV-specific peptides or both, by a high antibody affinity to BDV, or repeated seropositive results from the live-trapped, free-roaming vole (III).

Table 9. *Borna disease virus (BDV) antibodies in Eurasian wild rodents as detected by immunofluorescence assays using BDV strain He80 as the antigen.*

Panel	n	BDV-positive	Prevalence, %	CIs for prevalence[a]	Publication
Finland, total	**1146**	**4**	**0.35**	***0.095 – 0.89***	
South	57	1	1.75	*0.044 – 9.39*	Unpublished
West	530	1	0.19	*0.005 – 1.05*	III, Unpublished
North	130	0	< 0.77	*0 – 2.80*	III
East	36	0	< 2.8	*0 – 9.74*	III
Rodents near seropositive horses	393	2	0.51	*0.062 – 1.83*	III
Germany	**168**	**0**	**< 0.60**	***0 – 2.17***	Unpublished
Buryatia	**250**	**0**	**< 0.40**	***0 – 1.46***	Unpublished

[a] 95% confidence intervals, Fisher's exact test

Despite the recent BDV detection in the bicolored white-toothed shrew (*Crocidura leucodon*) in an endemic region in Switzerland (150, 252), BDV antibodies were not found in the 115 insectivores included in this study (data not shown, Kinnunen et al. unpublished). The tested shrews belonged to a different species (*Sorex araneus*) from the BDV-positive ones, and did not originate from a targeted, known endemic region, unlike the Swiss shrews, but were trapped as a part of other small mammal studies. As for rodents, BDV-antibody-positive voles were found in this study, indicating that BDV may have distinct hosts in different geographical areas. Apart from western and

north-western Germany, the geographical distribution of bicolored white-toothed shrews comprises all classical BDV-endemic regions in central Europe, as well as Israel and Turkey (288), but does not comply well to the current BDV distribution data in northern Europe (78, 299, 339). In contrast, the bank vole distribution is wider and covers all the European countries from which BDV infections have been reported (10). Thus, bank voles, together with bicolored white-toothed shrews as reservoirs, could explain the geographical pattern of BDV infections at least in Europe.

In Finland, BDV-infected rodents were found sporadically in the south, west and north (the latter near to a seropositive horse; III), with similar prevalences when the 95% confidence intervals are compared. Rodents from Buryatia and northern, non-BDV-endemic Germany did not harbour BDV-specific antibodies. Whether BDV infected wild rodents exist elsewhere in Germany remains to be determined, but this study clearly proves that wild voles may exhibit a BDV-specific antibody response indicating a natural infection. As is common in natural infections (119, 164, 271), at least three times more rodents tested had borderline results, probably indicating an antibody level around the IFA detection limit, suggesting that the real BDV prevalence may be higher than that interpreted from the obviously BDV-antibody positive rodents.

5.7 OPV DNA IS RARE IN RODENT ORGANS

Having detected OPV antibodies in wild rodents, PCRs, antibody measurements and virus isolation were applied to determine, how commonly the rodents are acutely infected, and furthermore, which OPV species are responsible for the antibodies. Despite the common OPV antibody findings in Eurasian wild rodents (Table 8), PCRs verified OPV DNA infrequently and in low amounts: none of the 156 Buryatian, and only 1 of 160 Finnish and 2 of 197 German rodents had OPV DNA with high C_t (cycle threshold) values (II). Cell cultures and suckling mice infected with these DNA-positive rodent tissues remained virus-negative, further suggesting a low OPV amount in the rodents (II). These findings of a high antibody but low DNA/virus prevalence demonstrate short-term OPV presence in wild rodents. This parallels the common finding of highly avid antibodies in 78% of the studied individuals, indicating that the majority of the detected OPV infections were not acute (II). In populations studied longitudinally, periods have, indeed, existed with very few acutely CPXV-infected rodents (25, 26).

In comparison to the longevity of the OPV antibody response (173), CPXV itself is only present for 1–5 weeks in wild rodent tissues (63, 202). Thus, for virus circulation, the transmission has to be effective during the short period of virus excretion or the virus must survive in the environment. Indeed, VACV, a correspondingly stabile, close relative to CPXV (199), maintains its titre in vaccine baits at ambient temperatures for at least one month (237) and endures in storm water for 56–166 days in 4 °C and

3–50 days at room temperature. It is also stable on natural products, namely salad, bread and sausage, for at least 14 days (97). Furthermore, VACV is stable in the faeces of VACV-infected mice at room temperature for 20 days (1). Such viral stability is certainly sufficient for CPXV to also be indirectly transmitted to another rodent via the environment, which might be especially crucial for viral circulation during cool winters when the virus survives best, the rodent population density is low and rodents have little direct contact. Thus, transmission cycles may differ according to the season and the phase of rodent population dynamics. CPXV transmission probably also differs between species (307) and the functional rodent groups possessing different sex and maturity statuses: for instance, adults may become infected more commonly in direct contact when mating or fighting, and subadults indirectly as well as by horizontal transmission. This hypothesised indirect transmission may explain how CPXV can exist in populations despite the rarity of active infections. Indirect transmission has not been included in the mathematical CPXV transmission models, which have shown that the transmission function in the field vole is something between density and frequency dependent, but not clearly either of them (292).

In the current study, the overall OPV-PCR prevalence was low in rodents, but acute infections could be demonstrated in as many as 25% (3 of 12) of the locations with antibody-positive individuals, as evidenced by DNA positivity (II).

5.8 FINNISH CPXV VARIANT RESEMBLES A RUSSIAN ISOLATE

The OPV PCR amplicons from patients and rodents were sequenced and the sequences analysed to clarify the virus species and their molecular epidemiology. From the viruses T2000 and E1989, isolated from the Finnish cowpox patients, 948-bp-long, identical haemagglutinin (HA) gene sequences were obtained. They matched 96 to 97% to other CPXV isolates and grouped in a phylogenetic tree, irrespective of the method, together with a Russian isolate GR-90 (I; (203)). The Finnish and Russian CPXVs formed their own clade, separate from other OPVs.

These findings have been recently verified and expanded: in addition to the HA gene, FIN/T2000 also clusters together with GR-90 according to the sequence of the CHOhr gene (132). The same study has furthermore confirmed with several strains the finding of this (I) and other work (120, 193): CPXV strains are mainly scattered among other OPVs in phylogenetic trees and do not comprise a separate CPXV clade. The results of one study, however, provided some evidence of geographical clustering, as Finnish and Russian strains, as well as all but one Norwegian and Swedish strains, respectively grouped together (132). The close relationship of the Finnish and Russian isolates suggests their connected evolutionary histories.

Interestingly, the HA sequences of the two cowpox cases 11 years apart, as well as the 179-bp-long sequence stretch from the Finnish bank vole, were fully identical to each other, indicating that a single CPXV distinct from the other Scandinavian and central European isolates may circulate in Finland (I, II). In this respect, the Finnish genotypes seem to resemble their British more than their German and Norwegian counterparts, because more genetic variation is seen in CPXV genotypes in the latter two (132, 172, 214, 220, 243). This variation, but also the close relationship of all OPVs was seen when the 132-bp-long OPV sequences from German rodents were compared to other available OPV sequences: they were identical to some MPXVs and also to a few feline and human CPXV isolates from southern Germany (172), but were distinct from most other OPVs, including other German CPXV isolates (II). Although more sampling and longer sequences are needed to verify it, these OPV sequences from rodents most likely represent CPXV (see "5.6 OPV antibodies are common and BDV antibodies occasionally found in Eurasian wild rodents").

Preliminary data suggest that the Russian GR-90 isolate, which was closest to the Finnish isolates, is more pathogenic to mice and rats than the more distant Brighton and Turk-74 isolates (203). The possible clinical relevance of the sequence differences nevertheless remains to be shown.

5.9 BDV GENERALLY INFECTS BANK VOLES WITHOUT PATHOLOGICAL CHANGES, MAJOR SYMPTOMS OR AN ANTIBODY RESPONSE

Newborn bank voles were inoculated i.c. with BDV, observed for 2–8 weeks and studied with RT PCRs, (immuno)histology and IFA to determine, whether they become infected with BDV and how possible infection manifests. RT PCR and immunohistology demonstrated that BDV infects bank vole brains productively (IV). Despite the wide spread of the virus and high antigen amount in all 41 infected brains, only 7 animals showed focal, mild leptomeningoencephalitis. Moreover, 2 suffered from a loss of Purkinje cells (PC) via either necrosis or apoptosis, which was confirmed by the detection of active caspase-3 occasionally in PCs (161). The latter two voles were symptomatic, expressing either circling and falling or intermittent tremor, while the former finding of inflammation did not correlate with any clinical signs. Similar slight inflammatory reaction can occasionally be seen in BDV-infected laboratory rodents (156, 170, 250, 272, 337), and the loss of PCs is common in neonatally infected rats (337). In this study, other voles exhibited no pathological changes. Moreover, the majority (31 voles) of the infected and all the control (9) voles remained symptomless; the exceptions showed either hyperactivity (6) or neurological symptoms (2) or both (2; IV). With this animal number, however, no statistical support existed for the correlation of the clinical signs with BDV infection ($p = 0.055$, MidP exact test). Consistent with infected rats and indicative of the potential role of sexual hormones

in the pathogenesis (250), female sex was nevertheless a significant (p = 0.0154, Mid-P exact test) risk factor for the clinical signs. Locomotor hyperactivity similar to some of the voles was observable in BDV-infected MRL/+ mice (268). Although immunocompetent rats mostly die of the disease, neonatally infected rats remain symptomless —like the voles— except for slight learning difficulties (250). The fact that BDV only exceptionally kills bank voles and mainly causes no clinical signs at all is consistent with this species being a possible reservoir. Namely, the reservoir species cannot be too severely affected to be able to spread a pathogen.

The pathogenesis of BDV infection probably also depends on the viral strain (145) and passage; the virulence seems to increase by passaging in rodents (233, 268). We used the 4th rat passage of the laboratory strain He80, referred to as "rat BDV", originally isolated from a Borna-diseased horse but now rodent-adapted (273, 299). This strain has previously caused neurological disease in 7 of 8 and encephalitis in all infected rats (273). Despite this obvious virulence, the pathology and symptoms in bank voles remained negligible or absent. Whether further passages in the bank vole would increase the virulence in this particular species remains to be shown.

A detectable level of BDV-specific antibodies existed in 41% of the bank voles, with antibodies appearing after 3 or more weeks p.i. (IV). This seroconversion time correlates well with other experimentally infected rodents: the first i.c.-infected Lewis and hybrid rats become seropositive around 10 dpi, whereas black-hooded rats show an antibody response only from 35 dpi onwards (108, 147). However, after intranasal infection the Lewis rat seroconverts a little later, from 20 dpi onwards (216). All mouse strains develop BDV antibodies by 31–49 dpi when infected as weanlings (95, 268). Thus, the first antibody finding at 3 weeks p.i. is close to the average for laboratory rodents, but the common lack of antibodies even in those tested 6 weeks p.i. (IV) was surprising, as all but one of the laboratory rodents had previously mounted an antibody response (273). Such differences may relate to the fact that bank voles represent a wild animal population most likely having fully functioning immune systems – which is not always the case in laboratory-bred rat and mouse colonies (206, 217, 264). The ubiquity of antibody-negative but BDV-excreting bank voles should be taken into account when interpreting the BDV prevalences of wild rodents (e.g. in III), as when estimated based on IFA, the BDV prevalence in bank voles is underestimated (IV).

5.10 BDV SPREADS NEURONALLY THROUGH THE BANK VOLE BODY AND IS EXCRETED

Having verified that the experimental i.c. BDV infection of the bank vole is productive, the infection kinetics was investigated using immunostaining of the vole tissues and RT PCRs for urine and faeces. BDV antigens were commonly expressed in the peripheral nervous system of the bank vole (IV). For example, axons in nerves

innervating the alimentary tract, skeletal muscle, salivary gland, and urinary bladder were commonly BDV-positive, and occasionally also a few muscle cells in the urinary bladder (Fig. 14), skeletal muscle and heart. The peripheral spread could mainly be seen from week 4 p.i. and thereafter, but occasionally already happened 2 weeks p.i. Furthermore, BDV RNA was demonstrated in urine (3 voles), faeces (17 voles) or both (3 voles) of the infected but not in the control animals, indicating viral excretion after centrifugal spread from the CNS. (IV)

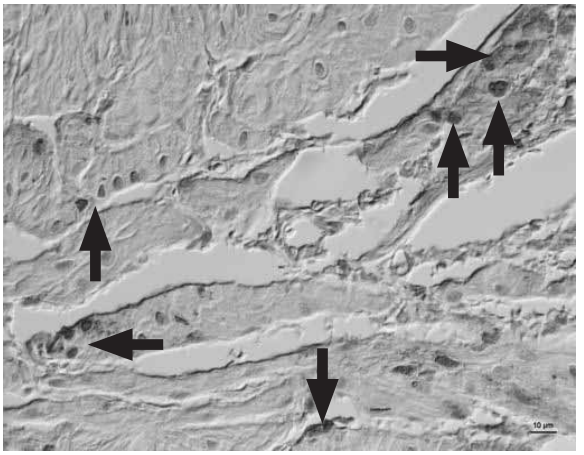

Figure 14. *Smooth muscle cells of the urinary bladder express BDV N-antigen in nuclei and cytoplasma (arrowheads). Immunostaining with mAb Bo-18 1:100. (Photo by courtesy of Anja Kipar)*

A peripheral BDV antigen distribution similar to bank voles can occur in i.c.-infected rats: newborn or immunoincompetent rats already show a peripheral distribution 14 dpi, compared to immunocompetent ones 28 dpi (303). In intranasally infected, immunocompetent rats, BDV spreads peripherally a little later, 38–60 dpi, although it is mainly restricted to their CNS (286). Recently, a peripheral nerve manifestation was also demonstrated in i.c.-infected mice, but very late, on 120 dpi (4). The peripheral spread of BDV in bank voles, already from 2 weeks p.i. on, more closely resembles that of rats in this respect (IV). In addition to the spread in peripheral nerves, laboratory rodents also excrete BDV abundantly in urine (10^3–10^4 50% tissue culture infective doses/ml, 500–10^4 ffu/ml) and less in tears or saliva (216, 273). Likewise, bank voles seemed to excrete BDV in urine, as evidenced by the common detection of RNA (17%; IV). Furthermore, BDV RNA existed even more commonly in their faeces (54%). Except for somewhat confusing PCR amplicons reported from three wild birds (36), no previous reports of BDV excretion in faeces have been identified. This result from the present work thus significantly widens current knowledge of BDV epidemiology. Like Puumala hantavirus, which bank voles excrete (134), BDV is peripherally spread and consecutively excreted in bank voles indicating that they could function as a BDV reservoir.

5.11 BDV RNA IS READILY TRANSCRIBED INTO DNA *IN VIVO*

Because the genes of ancient bornaviruses have been reverse transcribed and integrated into vertebrate genomes as their DNA counterparts (27, 155), the presence of BDV DNA in the experimentally BDV-infected bank voles was assessed to determine whether this phenomenon occurs nowadays. As many as 66% of the bank voles had BDV N DNA in brain tissue, as evidenced by PCR analysis of the extracted DNAs and further verification with nuclease treatments: DNAse but not RNAse digestion removed the PCR positivity in these assays performed without an RT step (IV). The nuclease digestions thus verified that instead of an unspecific amplification of RNA, the amplification indeed resulted from DNA. In addition to N DNA, P RNA had also been reverse transcribed *in vivo* as P DNA existed in one animal; borderline results were seen in several others. Both N and P DNA were most common at 4 weeks p.i., although they could be seen from 2 to 8 weeks p.i. No BDV DNA was detected in the mock-inoculated bank voles (IV). These findings parallel, confirm and extend the recent observations of BDV N DNA in vertebrates (27, 155): exogenous BDV RNA is indeed reverse transcribed into DNA *in vivo* during infection. Notably, further studies will determine whether BDV DNA in experimentally infected bank voles is episomal or integrated into chromosomal DNA.

The genome of the bank vole is not yet available to determine whether it contains endogenized BDV-like elements (EBL), as in some other rodents (27, 155). EBLs, especially the most commonly found EBLN, might play a role in the epidemiology of BDV, being possibly advantageous and probably enabling a species to function as a reservoir. Based on the BDV literature, it has been hypothesised that species containing EBL sequences are more likely to be resistant to a deadly infection than those that do not (27). This might result from protection mediated by the expression of indigenous BDV N or other components. Indeed, the nucleocapsid components of BDV, i.e. N, P and X proteins, convey resistance to BDV infection in cell lines (113). Furthermore, specific N epitopes are crucial for sufficient activation of the CD8+ T lymphocytes mediating early control of BDV in rats (108). The delicate balance between pathology and protection depends on these particular CD8+ T cells (20, 108), thus warranting further studies in the light of recent EBLN (and other EBL) findings (27, 155) and also sequence determination of possible reservoir species, including the bank vole. As an important step on the way to understanding the endogenization of RNA virus sequences, the results here demonstrated that the reverse transcription of BDV RNA is common in live animals (IV).

5.12 BDV OR A BDV-LIKE VIRUS INFECTS HUMANS IN FINLAND

Serum samples from Finns were collected and assayed for antibodies indicative of BDV infection. IFA based on the BDV/He80 strain revealed three antibody-positive humans (III). One of them belonged to a panel consisting of veterinarians (138 sera), while the two others originated from a serological panel comprising patients suspected to have hantavirus infection (361 sera). The antibody positivity of the veterinarian was carefully verified: the antibodies were highly avid, recognized multiple antigenic BDV peptides and the recombinant BDV P-protein, and the veterinarian again tested positive in a blinded assessment with IFA based on a new sample eight years afterwards (III and Kinnunen et al. unpublished). Furthermore, the results could be reproduced in an independent laboratory. However, no BDV RNA could be detected in the veterinarian's whole blood (Kinnunen et al. unpublished). One of the antibody-positive individuals was not as thoroughly investigated due to the single low-volume sample obtained; therefore, a possibility remains that a BDV-like virus caused these serological findings. As the human genome does not contain endogenized BDV P sequences (27, 155), the reaction of the two tested BDV-antibody positive human sera with BDV P protein excludes the unlikely chance that the reaction in IFA was caused by autoantibodies against putatively translated EBLNs (III). Similar continuous BDV-IFA antibody positivity has recently been seen in psychiatric patients in Germany, but updating of the method during the study brought uncertainty to the interpretation of the result (142).

This extremely well verified BDV antibody positivity of a human, which reliably indicates a human bornavirus infection, is important because of the controversial interpretations of the commonness of human BDV infections and the existence of human Borna disease (45-48, 89, 90, 105, 106, 190, 191, 194, 227, 248, 271, 284, 298, 299, 308, 343). Therefore, neither the sample number nor the sensitivity of the assays was maximised in this study, but instead, the aim was to find verifiable infections. The veterinarian of this study is the best-characterised BDV-seropositive human so far, and together with the unambiguous detection of BDV antigen and RNA in a few human brain samples (83), it evidences that BDV or a closely-related bornavirus infects humans at least occasionally and does not always lead to a disastrous outcome.

6. CONCLUDING REMARKS

This thesis shows that the two zoonotic viruses, CPXV and BDV and/or their close relatives exist in Finland and infect several mammals, including wild rodents, domestic animals and humans (I–III). Furthermore, it extends the knowledge of their epidemiology and host spectrum (I–IV) as well as confirms and extends the recently found reverse transcription of BDV RNA into DNA in live animals (IV).

Wild rodents, especially the bank vole, are involved in the circulation of CPXV/OPV (I, II). Despite the rarity of active CPXV infections in the wild, the risk of zoonotic CPXV transmission from rodents exists widely in Eurasia, being occasionally locally very high. This represents an infection risk for domestic animals and humans, emphasising the importance of rodent control and personal hygiene during and after contact with rodents or their excreta. Although we are now better aware of the wide geographical distribution of CPXV, the likelihood of acquiring a CPXV infection has most probably not changed: the virus has been and will be in wild rodents. The individual risk of infection depends on the frequency of contacts with infectious hosts, as well as age, sex, and occupation, and probably also on the immune status (23). For an individual, the risk is nonetheless low, as the infectious virus is rare and not very contagious to humans or domestic animals. However, the severity of the outcome in the rare occasions when infection occurs has probably increased. Several factors have contributed to this increase: the lack of attenuating immunity caused by smallpox vaccinations in younger generations (I), the increased prevalence of exacerbating conditions such as allergic and atopic diseases (289), immunosuppressive viral infections and treatments, and old age. Consequently, the rare cowpox cases are more likely to be grave (I).

Based on the low seroprevalence, BDV is a rarity in Finland (III). However, 2–3 times more infected animals exist than can be deduced from the antibody findings alone (IV; (119, 164)). Thus, the virus is present and infects humans, horses, cats, dogs, and wild rodents, although the majority of infections remain undetected. As the reliable diagnosis of BD is not possible *intra vitam*, proper *post mortem* samples are needed to verify the true clinical relevance of BDV in Finland and elsewhere. During this work, the relevant detection methods were established and are ready for future use (III, IV).

Wild voles, especially the bank vole, are involved in the circulation of BDV and occasionally loosely associated with clinical cases at least in Finland (III). This research proved that bank voles can be productively infected i.c. with BDV (IV) and could therefore play a role in BDV epidemiology. However, infection via more natural routes should also be demonstrated, although the intranasal and i.c. routes resulted in similar symptom and pathological patterns in rats (60), so other routes of inoculation would

not necessarily bring more information. BDV is also transmitted from rat to rat (273), but not from mouse to mouse (95). Whether transmission occurs between bank voles (and shrews (252)) remains to be determined, but the general absence of pathological and clinical alterations, the common presence of BDV in the periphery resulting in excretion in urine and faeces (IV), and the serologically evidenced natural infections (III, Kinnunen et al. unpublished) clearly show the potential of bank voles to be a BDV reservoir.

Rodents, including wild rodents and some pets, carry and transmit several rodent-borne viral zoonoses (roboviruses) in direct or indirect contact with humans: hantaviruses such as Puumala, Dobrava, Hantaan, Seoul, Sin Nombre, Laguna Negra, Andes, and Black Creek Canal viruses; arenaviruses i.e. lymphocytic choriomeningitis virus, Lassa, Machupo, Guanarito, Whitewater Arroyo, and Junin viruses; encephalomyocarditis picornavirus; Sandfly fever phlebovirus; tanapox virus; as well as orthopoxviruses such as MPXV and CPXV (180, 209). In addition, rodents are important maintenance hosts in several vector-borne viral zoonoses such as tick-borne encephalitis (312). This study has revealed that the rodent host range of CPXV/OPV is wider than thought (II), and in addition has nominated BDV as a candidate for the group of roboviruses (III, IV) or, when the recent BDV detection in shrews is noted (252), for the newly described (144), wider epidemiological group of rodent- and insectivore-borne "rainboviruses". To better understand the rainboviral nature of BDV, infection and transmission experiments should be conducted in shrews.

What else could be the BDV reservoir other than voles and shrews? Because the temporal distribution (spring; (90)) does not entirely fit into the epidemiological picture of a rainbovirus, especially without incubation periods not shorter than 3 to 4 months, other mammals should not be too easily excluded from the list of potential reservoirs. Bats, in particular, host several related zoonoses of the order *Mononegavirales*, belonging to the families of *rhabdo-* (lyssaviruses), *paramyxo-* (Hendra and Nipah viruses), and *filoviridae* (Marburg and Ebola viruses). Plants, arthropods and birds have, in addition to mammals, been suggested as remotely possible BDV reservoirs (90). Although evidence exists for a plant virus having switched its host to a vertebrate (115), it is not very probable that a plant virus would infect mammals. Arthropods, including insects, are also probably not to blame, since BDV does not cause a high viraemia needed for the transmission of arboviruses, and experimentally BDV-fed ticks maintain BDV RNA for only 1–24 days ((275), according to (90)). Furthermore, if birds transmitted BDV in addition to ABV, BD would be more common, especially in Africa. Naturally, a publication bias might be partially responsible for the apparent absence of BD cases there. It is also possible that BDV has complex transmission chains involving several host or vector species or both. In addition to searching for BDV in new species, the timing of the peaks of primary infections (instead of the peaks of clinical cases) and their comparison with and plotting against likely host or vector numbers and activities would be crucial for elucidating the obscure transmission cycle

of BDV. Longitudinal sampling of susceptible sentinel animals with the detection of seroconversion or avidity measurement could be useful in the timing, as long as the incubation period is not properly known. Whatever the host and vector, it seems clear that the BDV transmission cycle needs it/them, as the virus is not easily horizontally transmitted, i.e. is not very contagious (75, 321).

Similarly to BDV, there are hypothetical hosts for CPXV infection other than wild rodents. This is an important issue, as in the majority of the human cowpox cases the infection source has been unknown (I; (23)). Bats, for example, could be studied for CPXV infection, as CPXV is ubiquitous in its host predilection, and the role of the dead bat remained obscure in the epidemiology of the cowpox eruption in the Finnish girl (See "5.2 CPXV can cause severe infection in unvaccinated atopic children"). Furthermore, mosquitoes mechanically transmit another poxvirus, myxoma virus (100), so why not OPVs in some cases? Insectivores may also be a reservoir, although shrews were seronegative in this study (Kinnunen et al. unpublished). Indeed, an intense mole contact probably launched clinical cowpox (203), and OPV antibodies have been found in common shrews (183, 317), but further studies are needed to elucidate whether they can be natural carriers instead of incidental hosts for CPXV. This study, however, demonstrated that CPXV/OPV infects a wide spectrum of rodent hosts, namely seven species (II).

According to the serological results, CPXV and BDV are probably under-diagnosed in Finland (I, III). Orthopox- and Borna disease virus infections can be effectively detected by the established methods (I–IV), and they should be remembered as differential diagnoses when encountering animal or human patients showing appurtenant symptoms. The declining prevalence of OPV protection, in particular, emphasises the need for public awareness, or at least among general practitioners and veterinarians. Several epidemiological studies have revealed that veterinarians, even more than other professionals working with animals, possess a higher risk to acquire a zoonotic infection (17), so they are in a crucial position to detect and consequently protect animal owners and themselves from zoonoses.

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